IN VITRO DEVELOPMENT OF DISSOCIATED AND IMMUNOMAGNETICALLY-PURIFIED EMBRYONIC CHICK OPTIC TECTUM CELLS

By

DENI SCOTT GALILEO

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN
PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1988

ACKNOWLEDGEMENTS

I would like to thank Dr. Paul Linser for both direction and yet ample latitude to explore and satisfy my own intellectual curiosities. I would also like to thank my committee comprised of Drs. Francis Davis, Carl Feldherr, and Chris West for guidance and constructive criticisms throughout my dissertation research. A special thanks goes to the University of Florida Division of Pediatric Hematology and Oncology and especially Dr. Adrian Gee for making this research possible through his commitment of continuous gifts of microspheres, antibodies, and encouragement to me. Also, I thank Dr. John Ugelstad of the University of Trondheim, Norway, for a generous gift of microspheres.

I would also like to thank Drs. Gudrun Bennett and Gerry Shaw of the University of Florida as well as Dr. Steve Pfeiffer of the University of Connecticut for gifts of antibodies without which most of this work could not have been done. Thanks are also in order to Drs. Paul Begovac and Steve Dworetzsky for many worthwhile discussions and sharing the common bond of being a usually helpless guinea pig in the experiment of education that runs continuously and often without controls in the big orange and blue apparatus. I whole-heartedly thank anyone that I have left out that has made my time in graduate school either intellectually rewarding, pleasurable, or

both. Alternatively, I wish to castigate those that have made the learning processes of myself and others at the University of Florida needlessly entangled in red tape, rules, regulations, grades, prejudices, position, rigidity, and everything else that runs counter to true learning.

Finally, I wish to thank Dr. Arlene Stecenko for friendship and encouragement for the past 3 years and for the ability to see that my quandaries could often easily be resolved.

TABLE OF CONTENTS

Page

ACKNOWLEDGEMENTS..... iii

LIST OF TABLES..... vi

LIST OF FIGURES..... vii

ABSTRACT.....viii

CHAPTERS

I INTRODUCTION (A NATURAL HISTORY)..... 1

II SEPARATION OF DAY 7-8 CELLS..... 4

Introduction..... 4
Materials and Methods..... 7
Results..... 19
Discussion..... 48

III SEPARATION OF DAY 12-13 CELLS..... 69

Introduction..... 69
Materials and Methods..... 72
Results..... 76
Discussion..... 99

IV CONCLUSIONS..... 115

REFERENCES..... 118

BIOGRAPHICAL SKETCH..... 124

LIST OF TABLES

Table		Page
2-1	Effects of substrata on monolayers.....	39
2-2	^{3}H-thymidine incorporation: Day 7-8 cells.....	49
3-1	^{3}H-thymidine incorporation: Day 12-13 cells....	98
3-2	^{3}H-thymidine incorporation: Monolayers.....102	

LIST OF FIGURES

Figure		Page
2-1	Chamber used for immunomagnetic separations...	13
2-2	Cell isolation in methylcellulose.....	21
2-3	A2B5 antigen modulation *in vitro*.....	25
2-4	*In vitro* development of A2B5(+) cells.....	27
2-5	A2B5 antigen modulation via methylcellulose...	30
2-6	Calculated incomplete separations.....	33
2-7	Remixed separated cells.....	35
2-8	Effects of substrata: glass vs polyornithine..	38
2-9	Neurofilament expression *in vitro*.....	43
2-10	GS expression *in vitro*.....	47
2-11	^{3}H-thymidine incorporation into monolayers....	51
2-12	Summary diagram of results.....	53
3-1	Markers for dissociated cells (graph).....	78
3-2	Markers for dissociated cells (photos).....	80
3-3	A2B5 antigen modulation *in vitro*.....	85
3-4	*In vitro* development of A2B5(+) cells.....	89
3-5	Filament expression *in vitro*	93
3-6	Oligodendrocyte development *in vitro*	97
3-7	^{3}H-thymidine incorporation into monolayers....101	
3-8	Summary diagram of results.....104	

Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

IN VITRO DEVELOPMENT OF DISSOCIATED AND IMMUNOMAGNETICALLY-PURIFIED EMBRYONIC CHICK OPTIC TECTUM CELLS

By

Deni Scott Galileo

August 1988

Chairman: Paul J. Linser
Major Department: Anatomy and Cell Biology

Immunomagnetic cell separation techniques were used to purify embryonic chick optic tectum cells from 2 different developmental ages for in vitro development studies. This negative cell selection method was based on reactivity of monoclonal antibody A2B5 with cell surfaces. Purified A2B5(-) cells obtained initially were >99.99% pure. Surprisingly, A2B5(+) cells rapidly appeared in the purified surface A2B5(-) cells in direct response to the immunomagnetic depletion. After 1 day in culture, levels of A2B5(+) cells in purified cultures equalled unpurified levels (≈50%). Similarly, visual densities of A2B5(+) neurons were equal in purified and unpurified long-term monolayer cultures.

Degeneration of purified cells on the neuron-selective substratum polyornithine suggested that these cells contained a paucity of neurons initially after separation. Immunohistochemistry combined with ^{3}H-thymidine autoradiography of cells and monolayers demonstrated that new DNA synthesis was required for neither the acquisition of surface A2B5-antigen, nor for differentiation into neurons. These results suggest that in early embryonic vertebrate brain (days 7 and 8) cells are present which are capable of replacing depleted neurons in vitro.

With day 12 and 13 cells, nearly all purified A2B5(-) cells were identifiable as glia by reacting with antibodies against either glutamine synthetase or galactocerebroside. Most (≈80%) of the purified A2B5(-) cells in culture for one day became A2B5(+). No increase in the percentage of A2B5(+) cells from 45% was observed in unpurified cultures. Long-term monolayer cultures from purified cells contained many A2B5(+) cells with a flattened glial or round morphology. These A2B5(+) cells frequently reacted with antibodies against glial fibrillary acidic protein and another glial marker, 5A11, which indicated a partial glial character. Additionally, flattened glial-like cells were found to contain elaborate networks of anti-neurofilament-M reactive filaments. The above combinations of markers were not found in unpurified monolayers and are believed to be a result of the immunomagnetic removal of neurons. It is

hypothesized that the abnormal phenotypes in purified cell cultures from day 12 and 13 cells represent unsuccessful responses of the glia to replenish depleted neurons most likely due to restricted developmental potentials.

CHAPTER I
INTRODUCTION (A NATURAL HISTORY)

The work presented in this dissertation is work that began as an attempt to study in depth the phenomenon of glutamine synthetase (GS) production in glia as mediated by neuronal contact. This enzyme is produced in embryonic chick retina cultures in glia that are in close apposition to neurons (Linser and Moscona, 1979). Subsequently, it was found that this phenomenon also occured in cultures of embryonic chick optic tectum cells (Linser and Perkins, 1987a) and probably occurs in the vertebrate central nervous system in general. To study this phenomenon directly, I wished to obtain purified glia that were not producing any GS to which I could add back neurons and trigger the gene expression at will.

I chose to utilize cell purification methods that employed antibodies as the means of discrimination between neurons and glia (immunoselection). I became aware of the immunomagnetic cell separation procedures that use small paramagnetic microspheres coated with specific antibodies to remove a "target" cell population from heterogenous populations. Although this method has been used in different forms in several different systems, it appears that it has most seriously been utilized by clinicians to

remove neuroblastomas and leukemias from human bone marrow with unparalled efficiency (Treleaven et al., 1984). Fortunately, one of only two laboratories in the United States that uses this method clinically is at the University of Florida in the Department of Pediatrics. Through the aid of a mutual friend (science is as human an endeavor as is anything else) I enlisted the aid of Dr. Adrian Gee, the scientist in charge of such bone marrow "purging" at U.F. He and his entire laboratory remained committed to me for supplying microspheres and antibody with which to coat them from the conception to completion of my research.

Since then much time was spent on developing a simple and effective procedure and separation chamber that would be suitable for the separation of embryonic brain cells. At first, it seemed that my procedure was not accomplishing separations since apparent target cells were always in what was hoped to be purified populations of nontarget cells. It then became quite clear that these target cells were appearing from nontarget cells after the separations. This in itself was a unique and unexpected finding. It was subsequently found that when different age embryonic cells were separated that the target cells which appeared in the purified populations apparently developed different phenotypes according to the age separated. Early embryonic cells appeared to be able to compensate for the depletion

of target cells (neurons), whereas older embryonic cells could not. Herein lies the natural division of my work into the two following chapters according to the results obtained: separation of day 7-8 cells (Chapter II) and separation of day 12-13 cells (Chapter III). Although these results precluded my ability to obtain purified populations of immature glia as I originally had hoped for, many unique and interesting experimental phenomena occured in the purified cultures. These have led to a better (or more confused, depending upon the point of view) understanding of the potentials and restrictions of embryonic cells when their development is perturbed in a controlled fashion.

CHAPTER II
SEPARATION OF DAY 7-8 CELLS

Introduction

During development of the chick optic tectum the various differentiated cell types emerge in a temporally stepwise but overlapping manner. The neurons are generally the first cell type to exit the mitotic cycle (LaVail and Cowan, 1971b) and to express a differentiation product such as neurofilaments (Bennett and DiLullo, 1985). The glia are generally later in becoming post-mitotic and show overt signs of differentiation (Linser and Perkins, 1987a) practically coincident with the completion of neurogenesis (LaVail and Cowan, 1971b; Fujita, 1964). This timing of overt differentiation, however, does not necessarily reflect the timing of when different cells are determined to become one cell type or another. Additionally, this general pattern does not necessarily imply when a cell is restricted in its ability to become anything else if its microenvironment were to change.

The mechanisms that govern cell determination and differentiation during brain development seem multiple but are poorly understood. Interactions between cells appear to influence development at several levels from physical positioning of neurons (Levitt and Rakic, 1980) to the

expression of specific glial gene products (Fisher, 1984; Linser and Moscona, 1979). Glutamine synthetase (GS), for example, is produced in glia in culture when the glia are in contact with neurons (Linser and Moscona, 1983; Linser and Perkins, 1987a; Wu et al., 1988). Obviously, to study a phenomenon such as this it would be of great advantage to be able to purify the immature glia so that neurons could be added back to elicit GS production. Such an ability could also in itself reveal other phenomena that involve cell interactions.

A major obstacle to studying interactions that take place during early development, such as those that lead to GS production, is that they occur when only few if any cells are identifiable by commonly recognized differentiation markers. Also early in development, most cells do not differ enough from each other physically to make use of such cell purification techniques as buoyant density centrifugation (Campbell et al., 1977; Sheffield et al., 1980). Methods that do not need overt physical differences for separation are those that utilize monoclonal antibodies that discriminate between the surfaces of different types of cells (immunoselection). Complement-mediated cell lysis has been used successfully with embryonic neural tissues (Politi and Adler, 1987; Nagata et al., 1986), but this method does not allow recovery of both cell types, and not all antibodies fix

complement. "Panning" (Wysocki and Sato, 1978) is an immunoselection method that allows for recovery of both cell types; however, the purity of cells obtained is marginally acceptable (≈95%) for most applications.

One negative separation method that utilizes monoclonal antibody binding to a target cell population to remove it from a mixed population is immunomagnetic purging. This method was developed for removing neuroblastoma cells from human bone marrow (Treleaven et al., 1984) and operates by attaching the target cells to paramagnetic polystyrene microspheres via antibody linkages and removing them with a magnet. Potentially all types of monoclonal antibodies against cell surface constituents can be used, and routine separations result in depletions of target cells by 4 to 5 orders of magnitude (Philip et al., 1987).

I have modified this method to work well with dissociated embryonic chick brain cells. In hopes of purifying immature glia for <u>in vitro</u> reassociation studies, I removed the majority of identifiable neurons by using the monoclonal antibody A2B5 (anti-ganglioside G_{D1c}; Eisenbarth et al., 1979). It was found that even though the initial purification of A2B5(-) cells was complete, by 24 hours in culture approximately 50% of these cells had become A2B5(+). This modulation of cell surface A2B5 antigen was found to be in direct response to the

depletion of A2B5(+) cells. Similarly, visual densities of A2B5(+) neurons and neurofilaments were equivalent in purified and unpurified cultures. New DNA synthesis was not required for either modulation of surface A2B5 antigen or differentiation of cells into neurons.

Materials and Methods

Animals

White Leghorn chick embryos were used throughout this study. Fertilized eggs were purchased from the Division of Poultry Science, University of Florida and stored at 15°C until initiation of incubation at 37.5°C in a standard humidified egg incubator. Time in days of incubation was used as the index of developmental age. For the present study, 7 and 8 day embryonic optic tecta were dissected at the tectal commissure and isolated free of non-neural tissues aseptically in calcium-magnesium free Tyrode's solution (CMF; Linser and Moscona, 1979).

Cell Culture

Dissociated cells were prepared by incubating tecta for 30 min. in 0.4% trypsin (Nutritional Biochemicals, Cleveland, OH) in CMF at 37°C, followed by dissociation with a Pasteur pipette in Medium 199 (Hank's salts, Degenstein formula; KC Biological, Lenexa, KS) containing 0.3 mg/ml soybean trypsin-inhibitor (Sigma Chemical Co., St. Louis, MO) and 0.03 mg/ml DNase I (Sigma) (SBTI-DNase).

Rotation-mediated suspension cultures of reaggregating cells were made by placing $2x10^7$ cells in 3 ml of Medium 199 supplemented with 10% fetal bovine serum (FBS; Gibco Laboratories, Grand Island, NY), 100U/ml penicillin + 100 µg/ml streptomycin sulfate (Gibco), and 10 µg/ml gentamycin sulfate (M.A.Bioproducts, Walkersville, MD). Cells were rotated in capped 25 ml Ehrlenmeyer flasks at approximately 75 rpm (1 inch radius) at 37°C in a rotary incubator (New Brunswick Scientific, Edison, NJ) and fed approximately every other day with the same medium. Two days previous to assaying cultures for glutamine synthetase (GS), cultures were fed with the above medium supplemented with 0.33 µg/ml hydrocortisone (Sigma). GS levels were assayed by the modified colorimetric method of Kirk as previously described (Linser and Moscona, 1979).

Adherent monolayer cultures were prepared by incubating 10^6 cells in 1 ml of the above medium in 24 well (200 mm^2) tissue culture plates (Corning Glass Works, Corning, NY). Cells were plated on either 1) the plastic itself, 2) inserts of 12 mm dia. round glass coverslips, 3) coverslips coated with 0.1 mg/ml poly-L-ornithine HBr (m.w.= 100,000; Sigma), 4) coverslips coated with polyornithine and then 1 mg/ml rat tail collagen (Sigma), or 5) inserts of 7.5 mil thick Aclar fluorohalocarbon film (Allied Chemical Corp., Morristown, NJ). Cultures were kept in a standard tissue culture incubator at 37°C in a 5% CO_2/

air atmosphere. Monolayer cultures were fed with fresh medium approximately every other day.

For cell isolation experiments, freshly dissociated cells were suspended in a semisolid medium of 1.4% methyl cellulose (Sigma) dissolved in the above culture medium at a density of 10^7 cells/ ml. Cultures were kept in loosely capped sterile Ehrlenmeyer flasks in a standard tissue culture incubator in a 5% CO_2 / air atmosphere. After different lengths of time cells were recovered from the semisolid medium by diluting the medium with several times the volume of Tyrode's solution and were collected by centrifugation.

Immunomagnetic Separations

Polyclonal sheep anti-mouse antibodies (kindly provided by Dr. Adrian P. Gee, Div. of Pediatric Hematology and Oncology, Univ. of Florida) used to coat the microspheres were prepared by hyperimmunization with purified mouse IgG (Organon Teknika- Cappell, West Chester, PA). Useful antiserum was obtained after 5-6 immunizations. Anti-mouse antibodies were affinity-purified on a column made from mouse IgG (Cappell) bound to Affi-Gel 10 (Bio-Rad Laboratories, Richmond, CA) and then mixed with Sepharose CL-4B (Pharmacia, Inc., Piscataway, NJ).

Cells used for immunomagnetic separation were rinsed in Tyrode's solution, incubated in 1/25 dilution of A2B5-conditioned hybridoma medium in Tyrode's with 10% heat

inactivated FBS (HI-FBS) for 30 min. at 4°C. Cells were then rinsed 2x in Tyrode's and resuspended in SBTI-DNase. The A2B5 hybridoma cell line was obtained from the American Type Culture Collection, Rockville, MD, through Dr. Michael F. Marusich, Univ. of Oregon, Eugene, OR. Cells were mixed for 30 min. at 4°C in SBTI-DNAse + 10% HI-FBS with paramagnetic polystyrene microspheres (4.5 μm; Dynal, Inc., Great Neck, NY) which were previously coated with sheep anti-mouse IgG prepared as above. A 15-fold excess of the number of microspheres/ the number of A2B5(+) target cells was used which corresponds to a 7.5-fold excess of microspheres/ total cells, since approximately 50% of dissociated cells were A2B5(+). The microspheres were ethanol sterilized then coated with 30-40 μg antibody/ mg microspheres in a concentration of at least 0.2 mg/ml antibody overnight at 4°C with rotation in a microfuge tube.

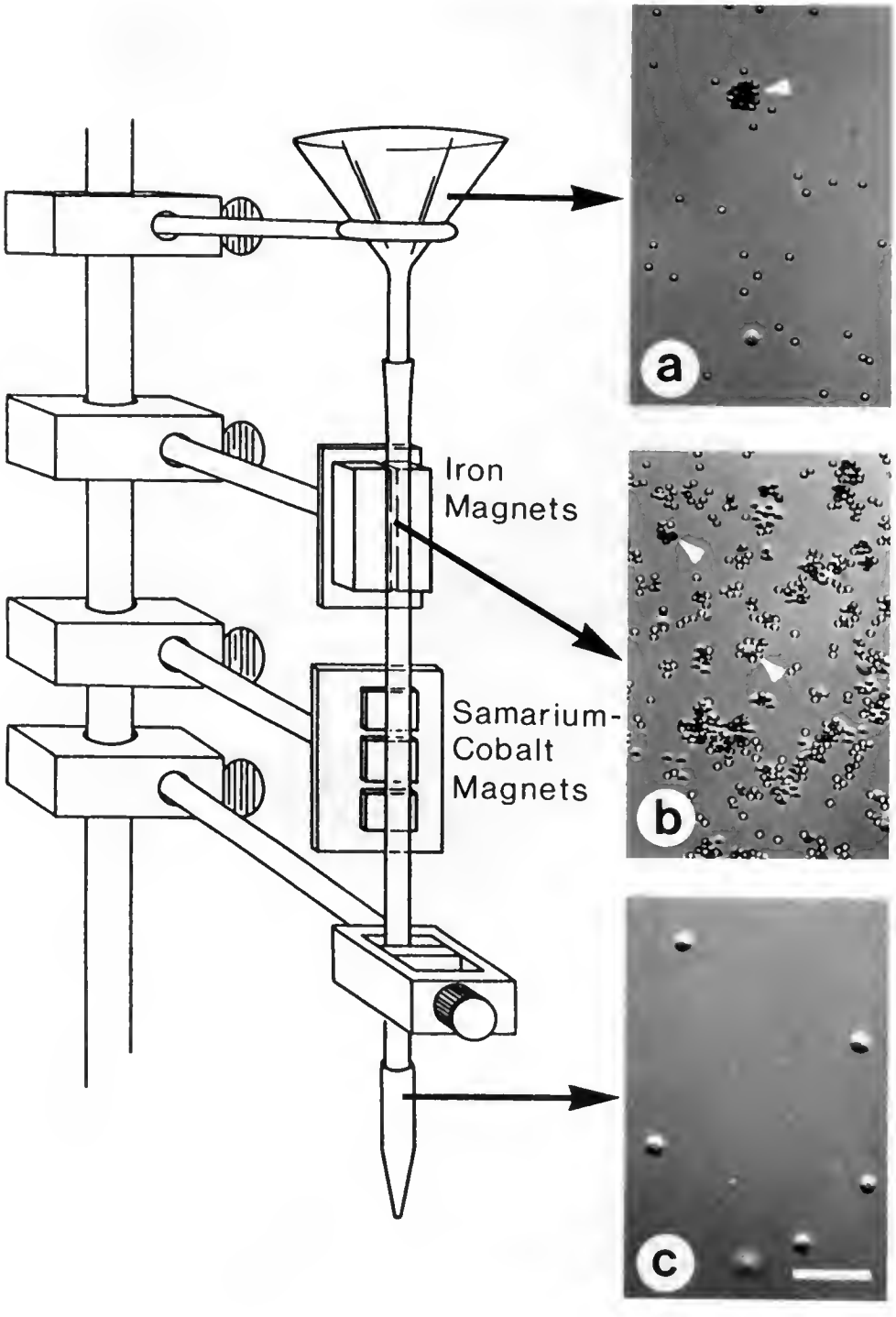
The cell-microsphere mix was then poured into the top of the separation chamber which was prefilled with Tyrode's, and unbound cells were eluted with Tyrode's by gravity at a rate of approximately 1-2 ml/ min. in a sterile hood until no more cells appeared in the eluant. In a typical experiment, approximately $3x10^7$ cells were separated in 30 min. The separation chamber was constructed from a plastic funnel and tubing (1/4" dia.) held on a ring stand (Fig.2-1). Two magnet arrays were held against the

side of the tubing, one made of ferrite magnets above one made of samarium-cobalt magnets. A pinch clamp at the bottom of the tubing was used to control the rate of flow induced by gravity.

Bound microsphere fraction cells were collected by first removing the magnet arrays from the side of the tubing and then washing out the bound cells and microspheres into a test tube. Cells were released from the microspheres by trypsinization as above followed by addition of SBTI-DNAse and vortexing. Freed microspheres were drawn away from the cells by placing the samarium-cobalt magnet array against the side of the tube and the suspended cells were aspirated out of the tube with a pipette.

Cells to be assessed for cell surface A2B5 following separation (approximately 2 hrs. after plating) were fixed in 1% formaldehyde (ACS grade, Fisher Scientific, Pittsburgh, PA) in phosphate-buffered saline (PBS) for 30 min., rinsed 3x in PBS, then incubated for 30 min. in 1/50 dilution of fluorescein-goat anti-mouse IgM (FITC-GAM IgM; Boehringer Mannheim Biochemicals, Indianapolis, IN) in PBS with 5% normal goat serum. Cells assayed for cell surface A2B5 after one day in culture were incubated live in 1/25 A2B5-conditioned hybridoma medium +10% HI-FBS on ice and then processed as above. Plating efficiencies of cells were determined retrospectively from Ektachrome slides by

Fig.2-1. Separation chamber used for the immunomagnetic separations and Nomarsky micrographs showing cells before and after the purification. Construction was of plastic tubing and funnel held on a ring stand. Two magnet arrays were used to ensure collection of all of the paramagnetic microspheres. A pinch clamp was used to control the rate of flow through the chamber. After ethanol sterilization in a sterile hood the cell-microsphere mix was poured into the funnel and the purified cells were collected in centrifuge tubes from the bottom. Micrograph (a) shows smaller microspheres binding to one cell (arrowhead) but not to another. Micrograph (b) shows cells that were removed from the mixture due to their coating of microspheres (arrowheads). Bottom micrograph (c) shows 6 purified cells without attached microspheres. Bar, 50μm.


Iron
Magnets
Samarium-
Cobalt
Magnets
a
b
c

counting the number of cells that adhered to the polyornithine coated coverslips initially after the experiment and after 1 day in culture. No attempt was made to determine cell numbers or densities in longer-term cultures.

Miniaggregate cultures were made of both unpurified and purified A2B5(-) cells in 24 well tissue culture plates on a substratum of 2% poly(2-hydroxyethyl methacrylate) (poly(HEMA)) (Interferon Sciences, Inc., New Brunswick, NJ) (Folkman and Moscona, 1978). This substratum prevented cell attachment to the plastic so that maximum intercellular contact and interaction could take place. Cultures were plated and fed as were adherent monolayers above. Two days before the GS assay was performed, cultures were fed with medium containing hydrocortisone as were rotation-mediated aggregate cultures.

Vital Dye Experiments

Experiments were performed which made use of the vital fluorescent carbocyanine dye DiO (3,3'-dioctadecyloxa-carbocyanine perchlorate; Molecular Probes, Eugene, OR) for cell marking purposes. These experiments were to determine if A2B5(+) cells would appear if less than all of the A2B5(+) cells were removed (incomplete separations), and also to determine if remixing the separated cells suppressed recruitment of new A2B5(+) cells (remixed separations). In both of these, cells that were to be

labelled were incubated in 200 µg/ml dye solution for 30 min. according to Honig and Hume (1986). DiO stained cells were viewed on an epifluorescent microscope with fluorescein optics and DiO fluorescence was not visible with rhodamine optics. Labelling efficiency with DiO was nearly 100%.

For calculated incomplete separations, part of the dissociated cells to be separated were incubated in A2B5 as in a normal separation followed by incubation in the dye DiO. These cells were then mixed with dissociated cells that were not incubated in either of these in some ratio (Fig. 2-6). This mix was then mixed with microspheres and separated in the magnetic column as above. Eluted unbound A2B5(-) cells were plated on polyornithine coverslips as described above. After one day in culture, these cultures along with control unseparated cultures were immunostained live with A2B5 as described above. A rhodamine-goat anti-mouse IgM (Fisher) secondary antibody was used to discriminate the DiO staining. The percent of the DiO(+) cells that were also A2B5(+) were scored.

In experiments where separated cells were remixed, the separation was carried out as normal (complete) and the bead-bound cell fraction was recovered via trypsinization of the beads (Fig.2-7). Eluted A2B5(-) cells were meanwhile incubated in DiO, and then the two separated cell fractions were remixed in even proportions. After one day in culture,

remixed cultures along with straight purified and control unpurified cultures were immunostained live with A2B5 and a rhodamine-goat anti-mouse secondary antibody as above. Scored were the percent of the DiO(+) cells that were also A2B5(+) in remixed cultures and percent A2B5(+) in purified and unpurified cultures.

Immunohistochemistry

Immunostaining with monoclonal antibody A2B5 was performed by incubating coverslips in 1/25 dilution of hybridoma supernatant in Tyrode's + 10% HI-FBS on ice for 30 min. Coverslips were then rinsed with Tyrode's 3x, fixed with 1% formaldehyde in phosphate-buffered saline (PBS) for 30 min., and rinsed 3x in PBS. Either fluorescein-goat anti-mouse IgM or rhodamine-goat anti-mouse IgM diluted 1/50 + 5% NGS in PBS for 30 min. was used as the secondary antibody. This procedure results in specific labelling of neurons in monolayer cultures without labelling the flattened glial cells.

Immunostaining of cells and monolayers with anti-galactocerebroside (GC) was performed essentially the same as with A2B5 above. A dilution of 1/50 of a purified monoclonal antibody against galactocerebroside (Ranscht et al., 1982; kindly provided by Dr. Steve Pfeiffer, Univ. of Conn. Health Center) was used. FITC-GAM IgG + IgM (Boehringer) was used as the secondary antibody.

For localization of GS in monolayer cultures, coverslips were reacted with A2B5 and then fixed in Bouin's fixative. Followed by rinsing 3x in PBS, cultures were then incubated in polyclonal antisera specific for GS (Linser and Moscona, 1979) at a dilution of 1/100 in PBS + 5% NGS for 30 min. After rinsing 3x in PBS, coverslips were incubated in a mixture of 1/50 FITC-GAM IgM and Texas Red goat anti-rabbit IgG (Fisher) + 5% NGS for 30 min. followed by rinsing 3x in PBS.

For localization of intermediate filaments, coverslip cultures were first immunostained with A2B5 and formaldehyde fixed as above. Cultures were then permeablized with 95% ethanol at -20°C for several minutes followed by rinsing in PBS. For glial filaments, cultures were incubated in a 1/50 dilution of polyclonal anti-glial fibrillary acidic protein (Dakopatts, Denmark) +5% NGS for 30 min. followed by rinsing 3x in PBS and incubation in the appropriate mixture of secondary antibodies. For neurofilaments, permeablized cultures were incubated in either a 1/250 dilution of polyclonal anti-neurofilament-M antiserum (Bennett et al., 1984) or a 1/100 dilution of a purified monoclonal antibody NF-1 (Shaw et al., 1986) followed by the appropriate mixture of secondary fluorescent antibodies.

^{3}H-Thymidine Incorporation

Incorporation of ^{3}H-thymidine was performed on both cells and monolayer cultures made from both unpurified and purified cells. Cells were plated on polyornithine coated 8 chamber Lab-Tek tissue culture chamber/slides (Miles Scientific, Naperville, IL) in culture medium containing 1 μCi/ml [methyl-^{3}H]-thymidine (6.7 Ci/mmol; NEN Research Products, Boston, MA) for analysis after 24 hours. For analysis of monolayer cultures, cells were plated on chamber/slides in the above medium and fed with fresh medium with label approximately every other day.

Cultures were rinsed in Tyrode's 3x, fixed in 1% formaldehyde, and immunostained with A2B5 as were coverslip cultures. Chamber wells and gaskets were removed and slides were dipped in Kodak NTB2 nuclear track emulsion (Eastman Kodak Co., Rochester, NY) diluted 1:2 with water. Slides were developed in Dektol developer (1:1) according to the manufacturer's directions after 4-5 days exposure at 4°C.

Micrography

All cultures were viewed and photographed using a Dialux 20 (Leitz, Switzerland) epifluorescent microscope equipped for mutually exclusive visualization of fluorescein and rhodamine fluorescence through a 40x Neofluar (Zeiss, West Germany) phase contrast objective which had a numerical aperture of 0.75.

Results

Cell Isolation in Methyl Cellulose

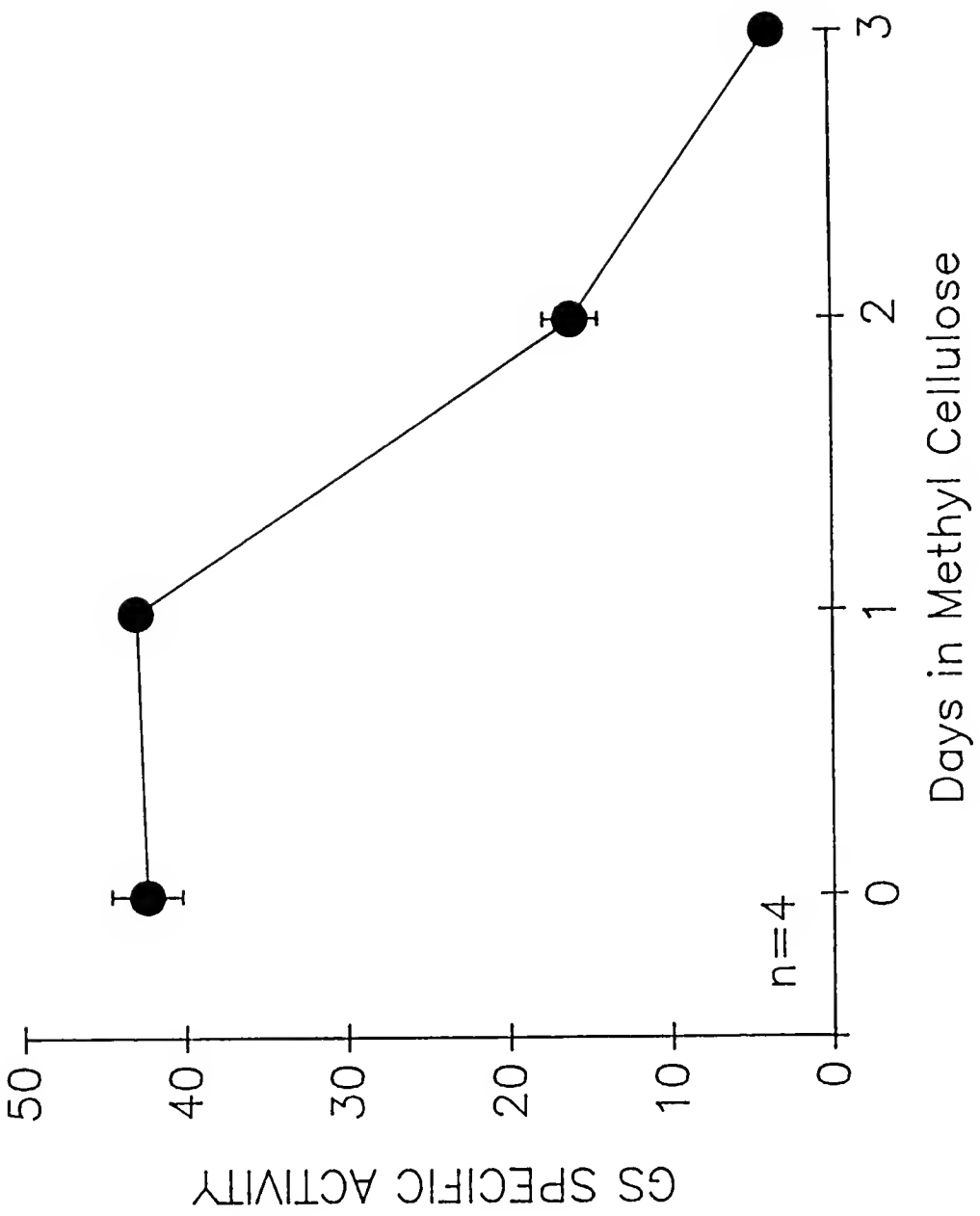
Using the tissue dissociation method above, cells were obtained that were nearly round and free of cell surface debris. Approximate yields were $2x10^7$ cells/ embryo or 10^7 cells/ lobe using 7 day and slightly higher ($2.5x10^7$/ embryo) for 8 day embryos. This yield was high compared to dissociations of tissue that is several days older (Chapter III). When suspended in the methyl cellulose, cells were at least several diameters apart from each other with no apparent physical contact.

The results of the effects of cell isolation on the production of GS after reaggregation are shown in Fig.2-2. GS was produced by cells isolated for 1 day at levels equaling those after immediate reaggregation. Thereafter, levels of GS fell rapidly, however levels were still measurable after 2 days of isolation. This demonstrated that these cells could be manipulated for up to 2 days before reaggregation was necessary if GS production was to be examined in culture.

Immunomagnetic Separations

Unless otherwise specifically stated, cell reactivity with A2B5 antibody refers only to cell surface binding and not to possible intracellular reactivity. The immunomagnetic cell separation procedure described herein produced initially extremely pure populations of A2B5(-)

Fig.2-2. Results of GS production after cells were suspended and isolated in methylcellulose medium for varying lengths of time. GS levels after one day in isolation were identical to that of cells which were reaggregated immeadiately. After longer periods of isolation the GS levels were reduced. Shown are the means ± s.e.m. (n=4).


GS SPECIFIC ACTIVITY
50
40
30
20
10
0
n=4
0
1
2
3
Days in Methyl Cellulose

cells from dissociated tecta. Purified cells were routinely >99.99% A2B5(-) as assayed by indirect immunofluorescence. Also, the vast majority of cells recovered from the microspheres were low level A2B5(+). Yields of A2B5(-) cells have ranged from 100% of theoretical yield (50% of total cells) to less than 50% of theoretical yield, depending on the batch of microspheres used. M450.40 microspheres gave the highest purified cell yields, while M450.51 and Dynabeads M450 gave significantly lower yields, presumably due to increased nonspecific binding to or trapping of A2B5(-) cells.

For separation of cells via A2B5, I have found it necessary to use a polyclonal antibody that was specifically produced for the purpose of microsphere coating. Several commercial affinity-purified polyclonal anti-mouse antibodies have been tested for effectiveness with this monoclonal without satisfactory purifications. Microspheres precoated with anti-mouse IgG (Dynal) have also been tested with similar unsuccessful results. The results presented here represent only experiments where the purity of A2B5(-) cells exceeded 99%.

A2B5 Antigen Modulation

Although the separations resulted in purified A2B5(-) cells, it was found that many A2B5(+) cells appeared after *in vitro* culture for only several hours. After one day in culture, approximately 50% of the initially purified cells

had become A2B5(+) (Fig.2-3). This is the same level of A2B5(+) cells in unpurified cultures after one day *in vitro*. Plating efficiencies of both unpurified and purified cells after one day in culture were nearly identical and ranged from approximately 85-100% as determined by retrospective counts from slides. Unpurified monolayer cultures (1-2 weeks *in vitro*) contained A2B5(+) cells that exhibited only neuronal morphologies that rested atop a layer of A2B5(-) flat cells either singly or in multicellular aggregates (Fig.2-4). Similarly, A2B5(+) cells with neuronal morphologies with a layer of A2B5(-) flat cells beneath them were present in monolayers from purified cells. The clusters of A2B5(+) neurons in both types of cultures appeared to be the same visual density under high or low magnification. No difference in the amount of A2B5(+) neurons in either type of culture was seen. The cultures made from microsphere cells, however, always contained visually larger and/or more numerous aggregates of A2B5(+) neurons than did the unpurified or purified cultures (not shown).

To assess the requirement of serum (FBS) in purified cultures where A2B5 antigen was modulated, purified cells were plated on polyornithine coated coverslips as above. After one day in culture, cells were immunostained live with A2B5 as above. To determine whether or not the modulated A2B5 antigen was trypsin-resistant as was the

Fig.2-3. Quantitation of A2B5(+) cells when unpurified and purified at the time of separation and after one day in culture. The unpurified cells show a small but significant increase in the number of A2B5(+) cells after one day. The purified cells are initially almost entirely A2B5(-), but after one day contain the same number of A2B5(+) cells as unpurified cultures. Shown are means ± s.e.m. for 4 individual experiments.

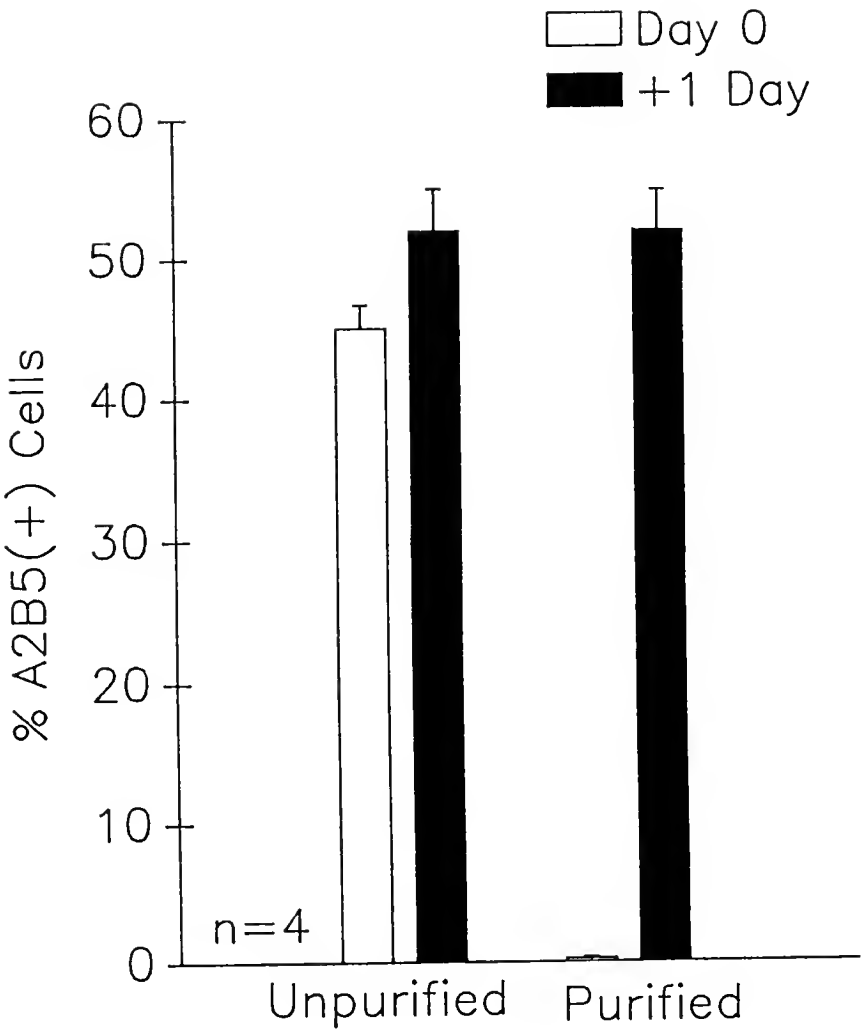
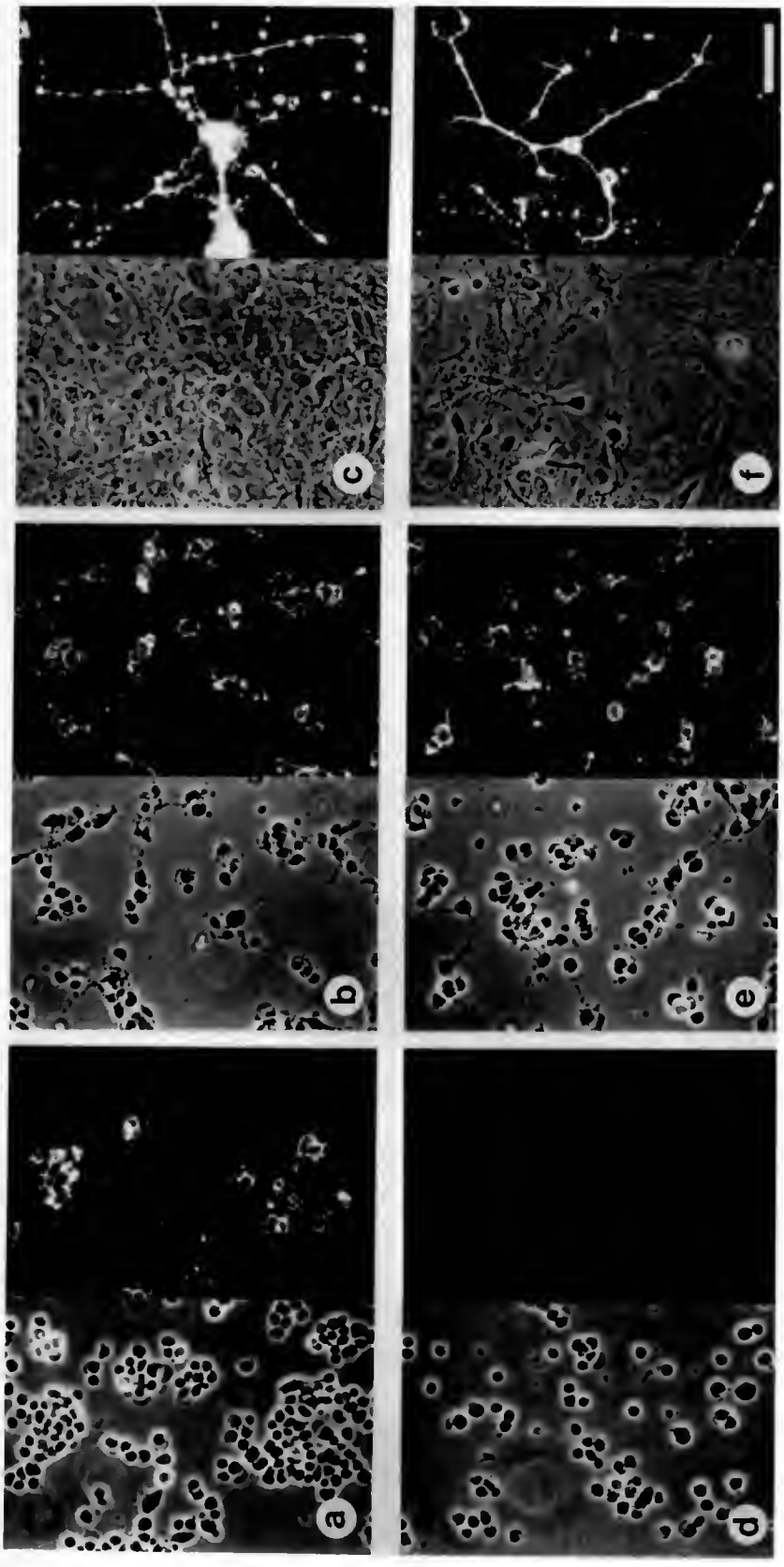

Day 0
+1 Day
% A2B5(+) Cells
60
50
40
30
20
10
0
n=4
Unpurified
Purified

Fig.2-4. In vitro development of A2B5(+) cells in both unpurified (a-c) and purified (d-f) cultures. Initially after the separation the unpurified cells (a) were ≈45% A2B5(+) while the purified cells (d) were devoid of A2B5(+) cells. After one day in culture both the unpurified (b) and purified (e) cells were ≈50% A2B5(+). Similarly, monolayer cultures after approximately 1-2 weeks contained A2B5(+) cells with neuronal morphologies. A2B5 did not react with the flat cells underlying the neurons. Shown are monolayers after 8 days in culture at high magnification. The fields of neurons shown in c) and f) were chosen to illustrate the morphologies of the A2B5(+) neurons and not to show the equal densities of neuronal clusters that were present. Bar, 50μm.


c
f
b
e
a
d

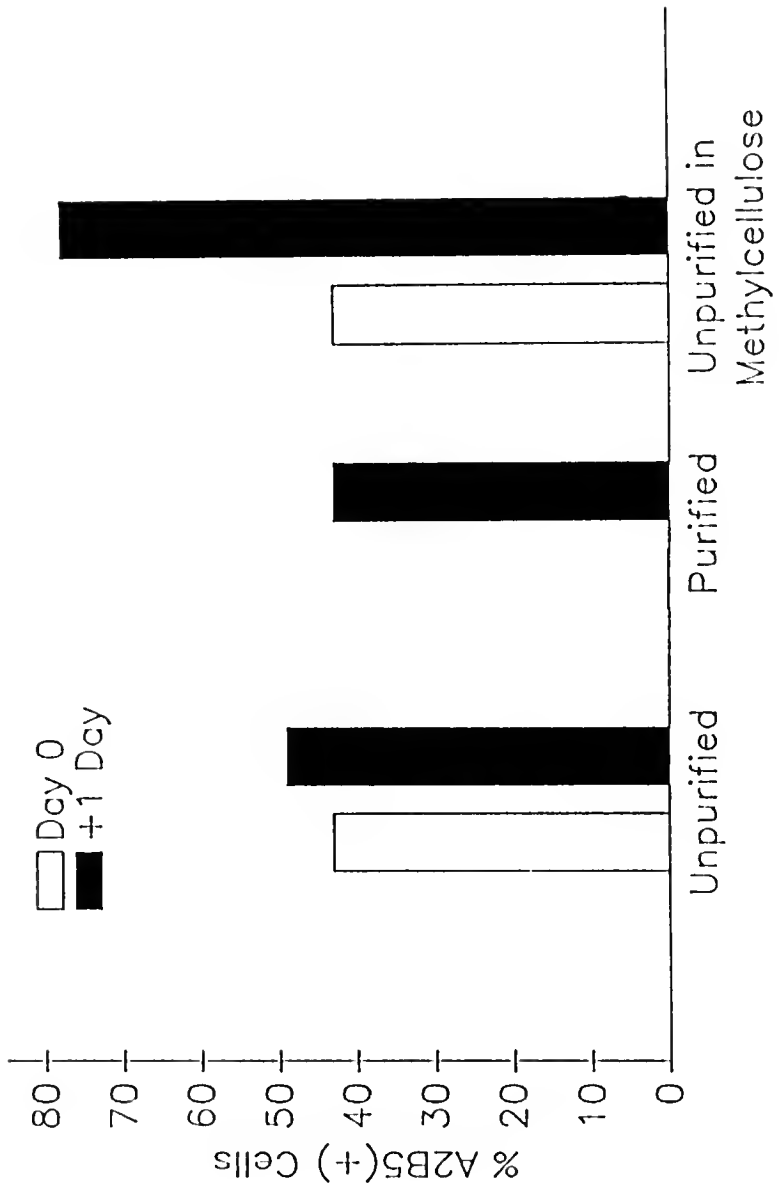
antigen in freshly dissociated cells, purified cells were plated on polyornithine coverslips as above. After one day in culture, coverslips were subjected to the same trypsinization regime as was used to dissociate tissue. Cells were then immunostained for surface A2B5 antigen as above. In both instances, it was found that there was no reduction in the percentage of A2B5(+) cells (≈50%). The experiments that entailed trypsinization, however, resulted in a large (unquantitated) release of cells from the coverslip due to the trypsin treatment.

Subsequent to the finding of the appearance of A2B5(+) cells in immunomagnetically-purified cell cultures, I predicted that an analogous increase in A2B5(+) cells may have occured when unseparated cells were suspended in methyl cellulose for a day. It was realized that suspension in methyl cellulose was in effect also a separation that would not allow the A2B5(+) and (-) cells to interact. This prediction was found to be true both qualitatively and quantitatively (Fig.2-5). Over 50% of the cells that were A2B5(-) when suspended in the methylcellulose converted to A2B5(+) after one day. Thus by two different techniques the ability of A2B5(-) cells to become A2B5(+) when isolated has been demonstrated with similar quantitative results.

Vital Dye Experiments

I made use of the a vital fluorescent dye, DiO, to label certain cell populations for either controlled

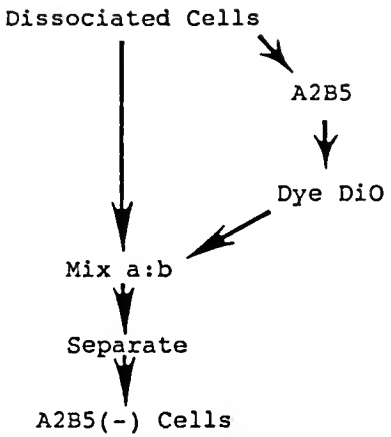
Fig.2-5. Quantitation of A2B5(+) cells initially and after one day in culture when unpurified, purified, and when suspended in methylcellulose. Results for unpurified and purified cells are essentially the same as in Fig.1-3. The cells isolated in methylcellulose for one day exhibited a large quantitatively similar increase in the number of A2B5(+) cells as did the purified cells (½ of the cells that were A2B5(-) on day 0).


% A2B5(+) Cells
0
10
20
30
40
50
60
70
80
Day 0
+1 Day
Unpurified
Purified
Unpurified in Methylcellulose

"incomplete" immunomagnetic separations or remixing experiments as diagrammed in Figs. 2-6 and 2-7. This was done to determine if new A2B5(+) cells would appear if only some of the A2B5(+) cells were removed, and also to determine if appearance of new A2B5(+) cells in purified cultures could be suppressed by adding back the cells that were removed. The results of the incomplete separations are shown in Fig.2-6. The abscissa is the percent cells incubated in A2B5 before separation which is in effect the percent of A2B5(+) cells removed, since the remaining cells had not been exposed to the monoclonal antibody. The ordinate expresses the percent of the known A2B5(-)/DiO(+) cells that became A2B5(+) after one day in culture. It can be clearly seen that the result of removing increasing numbers of A2B5(+) cells was increasing recruitment of A2B5(-) cells to become A2B5(+). Thus the increase of A2B5(+) cells in purified cultures was in direct response to the depletion of A2B5(+) cells.

Fig.2-7 shows the lack of effect of mixing back those cells that were removed with purified cells on the recruitment of newly appeared A2B5(+) cells. Here, complete separations were performed and the purified A2B5(-) cells were incubated in dye before remixing with cells recovered from the microspheres. It is clear that mixing back those cells that were removed had no effect on decreasing the amount of newly appeared A2B5(+) cells from those that were

Fig.2-6. Experimental design and results of controlled incomplete separations. Plain dissociated cells were mixed in some ratio with those that were incubated in A2B5 and then the vital fluorescent dye DiO (diagram). After the immunomagnetic separation cells were plated on polyornithine. After 1 day in culture cells were immunostained with A2B5 and scored were the % of DiO(+) cells that had become A2B5(+), except with the leftmost points where none of the cells were incubated in dye or A2B5. Those cells were scored for %A2B5(+) cells initially and after one day in culture. Plating efficiencies after one day in culture approached 100%. The results graph shows that as a greater percentage of the A2B5(+) cells are removed from dissociated cells, a greater percentage of the known A2B5(-) cells convert to A2B5(+) cells. If no A2B5(+) cells are removed then only a slight increase in A2B5(+) cells occurs. The (2) indicates two identical results at that point. The results indicate that A2B5(+) cells are recruited from A2B5(-) cells as a function of the depletion of A2B5(+) cells.



+1 Day
1) Immunostain with A2B5
2) Score % of dye(+) cells that are also A2B5(+)

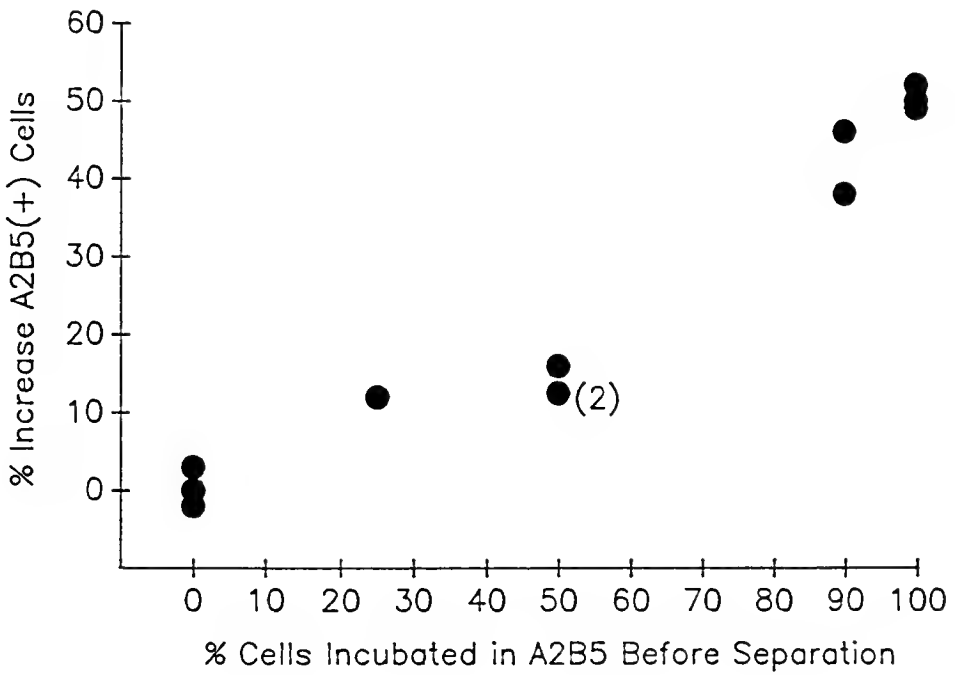
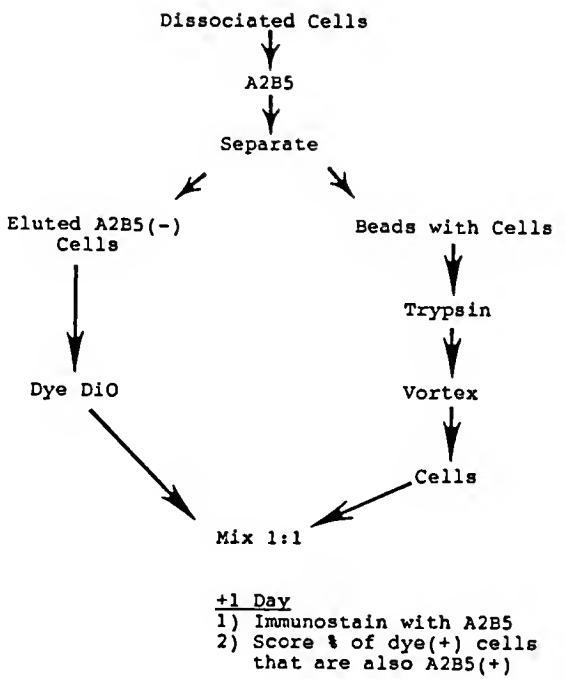
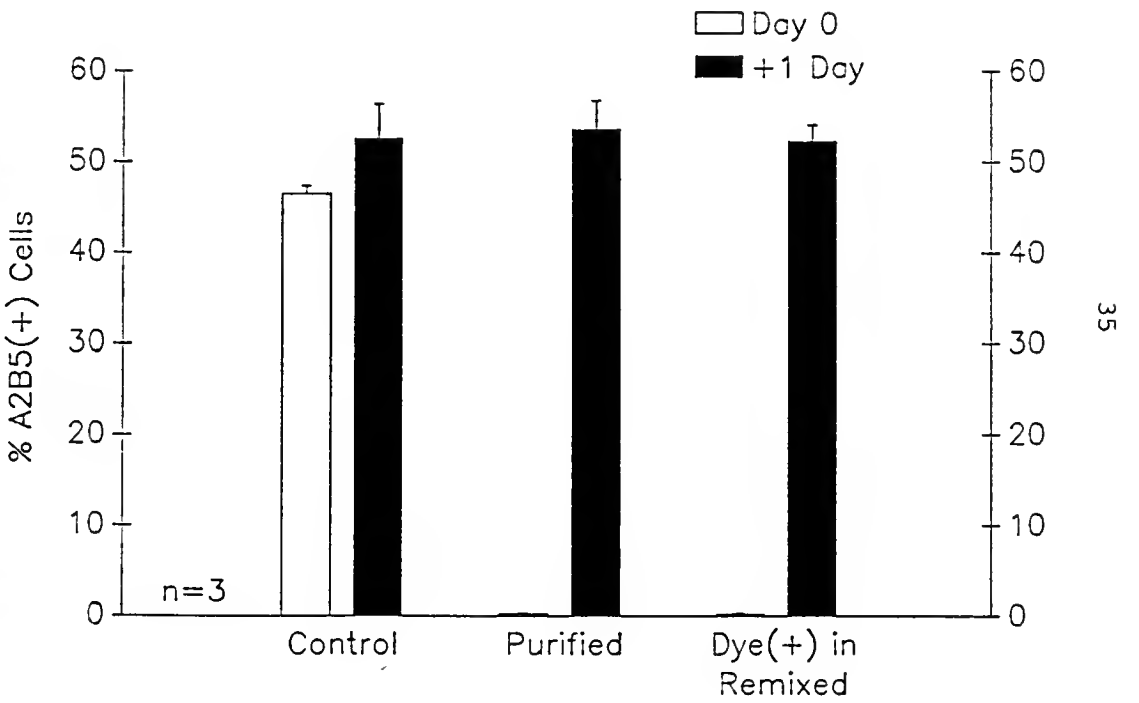


Fig.2-7. Design of experiments and results in which separated cells were remixed. Separation was carried out as usual. Purified A2B5(-) cells were incubated in the dye DiO and then remixed with cells that were recovered from the microspheres after trypsinization. After 1 day in culture the remixed cultures were immunostained with A2B5 and scored was the % of Dye(+) cells that had become A2B5(+). The results graph shows the effect of remixing separated cells on the recruitment of A2B5(+) cells from A2B5(-) cells. The results obtained with unpurified (control) and purified cells initially and after 1 day in culture are essentially the same as Fig.1-3. Remixing back those A2B5(+) cells that were removed during the purification had no effect on reducing the number of A2B5(+) cells that were recruited from A2B5(-) cells (rightmost graph). Shown are means ± s.e.m. for three separate experiments.


Dissociated Cells
A2B5
Separate
Eluted A2B5(-) Cells
Beads with Cells
Trypsin
Vortex
Cells
Dye DiO
Mix 1:1
+1 Day
1) Immunostain with A2B5
2) Score % of dye(+) cells that are also A2B5(+)


Day 0
+1 Day
% A2B5(+) Cells
60
50
40
30
20
10
0
n=3
Control
Purified
Dye(+) in Remixed

A2B5(-). Furthermore, when cells were allowed to develop into monolayers, A2B5(+) neurons that had retained the dye were found (not shown). Internalization of dye from cell surfaces as well as a high dye background from labelled flat cells made it impossible, however, to quantitate the percentage of A2B5(+) neurons that were dye labelled. Nonetheless, recruitment of A2B5(+) cells did not seem to be suppressable with this experimental regime.

Effects of Substrata

The effects of different substrata on the development of monolayer cultures was investigated to determine whether purified cells might develop differently than unpurified cells. Adler et al. (1979) showed previously that different substrata had profoundly different effects on the development of day 7 optic tectum cells. Here, when cells were plated on either plastic, glass, or collagen multicellular aggregates formed rapidly (<1 day) and then attached to the substratum after about a week in culture. Flat cells then migrated out of the aggregates on the substratum eventually forming a mature monolayer culture with networks of A2B5(+) neurons and neuronal aggregates growing on top of the layer of A2B5(-) flat cells (Fig. 2-8). This was true for both unpurified or purified A2B5(-) cells. These different substrata appeared to be totally nonselective for the growth of neurons and glia in culture (Table 2-1).

Fig.2-8. The effects of substrata on the development of monolayer cultures (11 days *in vitro*) of unpurified (a,e), purified (b,f), microsphere fraction (c,g), and remixed separated cells (d,h). Cultures shown were live when photographed. Monolayer cultures grown on either glass (a-d), plastic (not shown), Aclar (not shown), or collagen (not shown) substrata developed into a confluent layer of A2B5(-) flat cells on top of which was a network of interconnected aggregates (arrowheads) of A2B5(+) neurons. Cultures from (a) unpurified, (b) purified, and (d) remixed cultures contained the same visual density of neurons and clusters of neurons at both high and low magnifications. Cultures made from microsphere fraction cells (c) contained larger aggregates of neurons than the others. Monolayer cultures grown on polyornithine substrata (e-h), however, resulted in a network of neurons amidst nuclei from degenerated glia in cultures made from unpurified (e) and remixed (h) cells. Cultures made from purified A2B5(-) cells (f) completely degenerated. Cultures made from microsphere recovered cells (g) contained a denser network of neurons than from unpurified cells. Bar, 50μm.

Unpurified
Purified
Bead
Mixed
a
b
c
d
e
f
g
h

Table 2-1
Effects of substrata on monolayers[a]

Cells	Substratum	Growth
Unpurified	Glass	All cells
	Plastic	"
	Aclar	"
	Collagen	"
	Polyornithine	Neurons only
Purified	Glass	All cells
	Plastic	"
	Aclar	"
	Collagen	"
	Polyornithine	No growth
Microsphere	Glass	All cells
	Plastic	"
	Aclar	"
	Collagen	"
	Polyornithine	Neurons only
Mixed	Glass	All cells
	Plastic	"
	Aclar	"
	Collagen	"
	Polyornithine	Neurons only

[a] Effects of substrata on the growth of neurons and glia in unpurified and purified monolayer cultures. The only substratum out of those tested which was cell type selective was polyornithine which was selective for neurons. Practically all cells degenerated on polyornithine in cultures made from purified A2B5(-) cells.

The substratum polyornithine resulted in much different development of embryonic tectum cells in vitro. As observed by Adler et al. (1979), when dissociated day 7 or 8 tectum cells were plated on polyornithine only the neurons developed and the glia degenerated over a period of about a week. This resulted in aggregates of neurons with interconnecting processes attached to the coverslips amidst the nuclei and debris of dead glia (Fig.2-8). When purified A2B5(-) cells were plated on polyornithine virtually all cells degenerated. Conversely, when cells recovered from the microspheres (A2B5(+)-enriched) were plated on this substratum many neurons developed. In fact, this fraction of cells resulted in the most visually dense networks of neurons. This suggests that the purified A2B5(-) cells were deficient in neurons and that the microsphere cells were enriched in them.

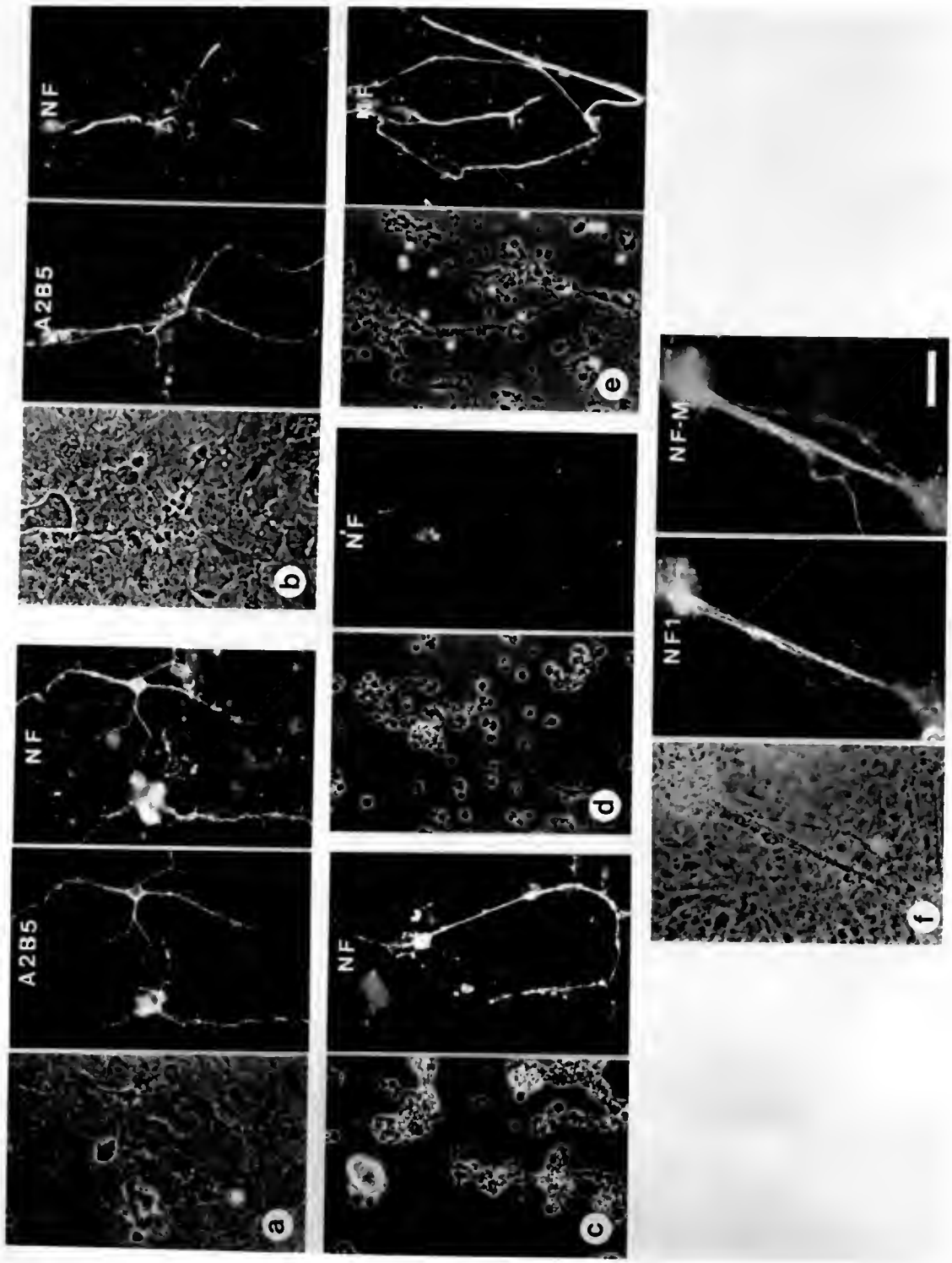
Filament Expression In Vitro

The expression in vitro of two neural tissue specific intermediate filaments was examined by immunohistochemistry of monolayer cultures. Glial fibrillary acidic protein (GFAP) positive filaments were found to be present in the population of A2B5(-) flat glial cells underlying the neurons (not shown). Immuno-reactivity was seen in most if not all flat cells but not in cells with a neuronal morphology. Unlike GFAP, there were cells present in dissociated 7 or 8 day tectum that reacted with antibodies

specific for neurofilaments (see Chapter 3). Twelve percent of unpurified cells showed immuno-reactivity with a polyclonal antisera specific for the phosphorylated form of the middle weight chicken neurofilament triplet protein (NF-M; Bennett et al., 1984). The pattern of staining was mostly filamentous surrounding the nucleus. This pattern is somewhat surprising since neurofilament reactivity was confined to cellular processes (axons) in tissue sections from the same age tecta (data not shown). This may suggest that the dissociation procedure caused a retraction of processes by neurons. When cells were double labelled with A2B5, the large majority of NF-M(+) cells were also A2B5(+). Twelve percent of the total dissociated cells were NF-M(+). Ten out of 12 of these cells were also A2B5(+), so 83% of the NF(+) cells were also A2B5(+). Alternatively, 2% of the total cells were A2B5(-) and NF-M(+), leaving 4% NF-M(+) cells in the purified A2B5(-) population.

Neurofilament expression was examined in monolayer cultures with both the polyclonal antisera and a purified monoclonal antibody (NF1) specific for the phosphorylated form of the rat heavy (200kD) triplet protein (Shaw et al., 1986). Many of the A2B5(+) cells with neuronal morphology in both unpurified or purified cultures contained neurofilaments as evidenced by both antibodies (Fig.2-9). Likewise, greater than 90% of neurofilament containing neurons were A2B5(+). Cell monolayers that were made from

Fig.2-9. Neurofilament expression in monolayer cultures grown on glass and on polyornithine for approximately 2 weeks. Shown are high magnifications to demonstrate morphologies of individual cells. Anti-neurofilament reactivity (NF-M) was found in neurons in both (a) unpurified and (b) purified cultures to approximately the same extent on glass. A large number of filaments were present along with NF-M(+) cell bodies in many A2B5(+) neurons. Many A2B5(+) neurons (<50%) in both cultures did not react with antibodies against neurofilaments. A small number of NF-M(+) neurons were A2B5(-). Cultures on polyornithine of unpurified (c) and (e) microsphere fraction cells revealed networks of neurofilaments similar to those on glass. Purified cells (d) degenerated on polyornithine and correspondingly contained a paucity of NF-M reactivity. Unpurified cell cultures (f) on glass that were double labelled with antibodies against both the heavy and middle weight triplet proteins (NF1 and NF-M, respectively) showed similar but not identical patterns of reactivity in neurons. Anti- (NF-M) appeared to react with more processes than did NF1. Neither antibody reacted with filaments in the A2B5(-) flat cells underlying the neurons. Bar, 50μm.


A285
NF
a
b
c
d
e
f
NF1
NF-M

microsphere fraction cells had visibly more dense neurofilaments and A2B5(+) neurons (not shown). In monolayers, more neuronal processes were found that contained filaments positive for the polyclonal antisera than for NF1, and processes that contained filaments that were NF1(+) were almost always NF-M(+) as well. Thus, the patterns of neuronal and glial intermediate filaments appeared identical in purified and unpurified cells when grown on a substratum of glass.

The pattern of neurofilament immunostaining was very different, however, in purified versus unpurified cultures when grown on a substratum of polyornithine (Fig.2-9). As stated above, only a network of neurons survived when unpurified cells were cultured on polyornithine. As in neurons cultured on glass, many of the neurons on polyornithine contained neurofilaments. Purified cultures, on the other hand, degenerated on polyornithine and contained a paucity of neurofilaments. The few cells that survived and produced neurofilaments (or had them at the start) were presumably those A2B5(-)/NF-M(+) cells (see above) that eluted in the purified population.

Galactocerebroside Expression In Vitro

A monoclonal antibody specific for the membrane molecule galactocerebroside (GC) was used to study the development of oligodenrocytes in cultures of purified and unpurified tectum cells. No GC(+) cells were present in

dissociated 7 or 8 day tectum. Similarly, no GC(+) oligodendrocytes appeared in cultures of either unpurified or purified cells (not shown). GC(+) cells appear by day 12 of development (see Chapter 3).

GS Expression In Vitro

The expression of glutamine synthetase was examined in both aggregate and monolayer cultures to determine whether or not purified and unpurified cultures were similar in this respect. A quantitative assay revealed that both purified and unpurified cells produced the same levels of GS when allowed to reaggregate on poly(HEMA) (Fig.2-10). Similarly, immunostaining for GS revealed the same staining patterns in both purified and unpurified monolayer cultures: GS was produced in glia under or near aggregates of neurons (Fig.2-10). Thus, the production of GS in glia in purified cultures parallels that of unpurified cultures both quantitatively and with respect to position to neurons.

DNA Synthesis In Vitro

The synthesis of new DNA was examined by combining A2B5 immunostaining with ^{3}H-thymidine autoradiography of both newly plated cells and of monolayer cultures. Newly plated cells were subjected to continuous labelling with ^{3}H-thymidine for 24 hours, followed by immunostaining with A2B5 and autoradiography. With both purified and unpurified cells only a small number of nuclei were labelled (<5%;

Fig.2-10. GS expression *in vitro*. Graph shows results of GS assay performed on miniaggregate cultures on poly(HEMA) in culture for ≈ 1 week. Unpurified and purified cultures produced identical levels of GS. Micrographs show immunohistochemistry of monolayers (9 days *in vitro*) for GS. GS was produced only in glia underlying neuronal aggregates in both (a) unpurified and (b) purified cultures. Bar, 50μm.

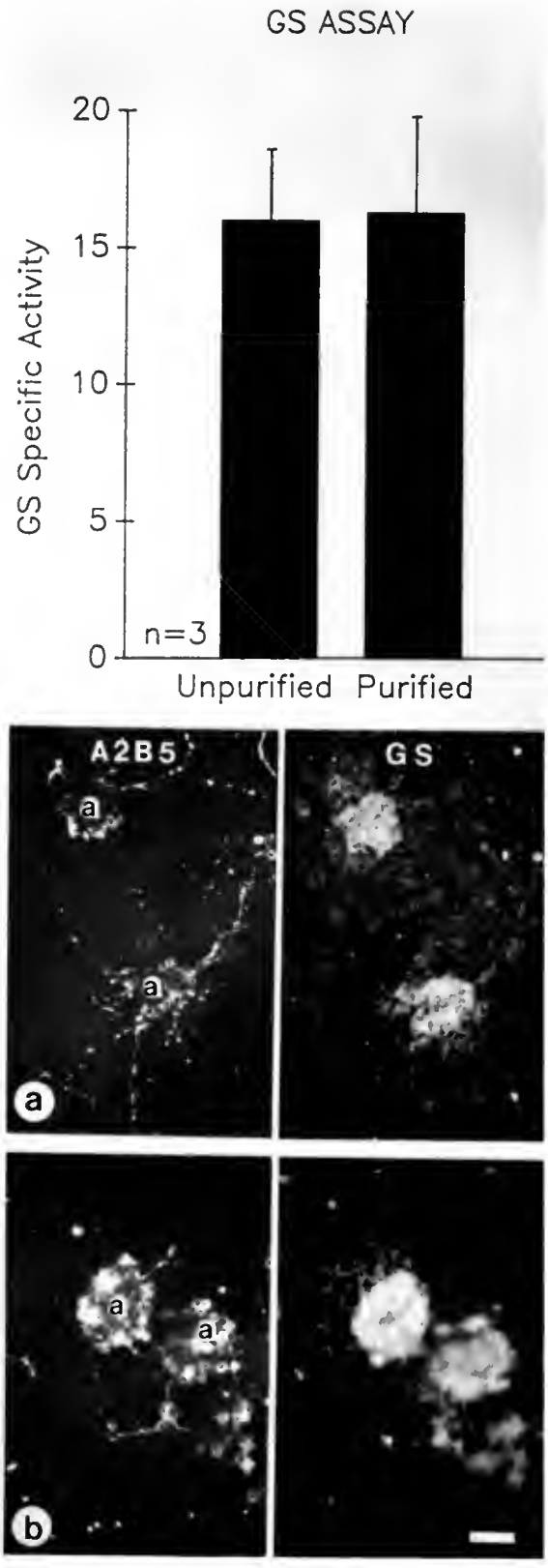

GS ASSAY
GS Specific Activity
20
15
10
5
0
n=3
Unpurified
Purified
A2B5
GS
a
a
a
a
a
b

Table 2-2) and all of these cells in both cultures were A2B5(-). See Fig. 2-11 for examples of labelled nuclei.

Similarly, long-term (1-2 weeks) monolayer cultures were subjected to continuous labelling with ^{3}H-thymidine followed by A2B5 immunostaining and autoradiography. The A2B5(+) neurons that developed in both purified and unpurified cultures did not contain labelled nuclei (Fig.2-11). Out of several hundred neurons examined where cell bodies could be clearly seen not one contained a labelled nucleus. The majority of A2B5(-) flat cells, however, had densely labelled nuclei. These nuclei were rather large in diameter, quite flat, and oval shaped.

Discussion

A diagrammatic summary of the results is presented in Fig.2-12. I have developed a method for the purification of dissociated embryonic brain cells based on the removal of a target cell population by specific antibody linkages to paramagnetic microspheres. This method was developed to purify embryonic glia so that phenomena such as the induction of GS by neurons could be studied *in vitro* in a controlled fashion. The cell isolation experiments in methylcellulose demonstrated that embryonic tectum cells could be manipulated for a significant length of time (≈2 days) before they lost competence for GS production when reaggregated. Thus it seemed entirely possible to develop a method of separation that would be useful within this time

Table 2-2
^{3}H-thymidine incorporation:
Day 7-8 cells[a]

Cells	Score	% Labelled Nuclei
Unpurified	21/519	4.0%
Purified	28/566	4.9%

[a] ^{3}H-thymidine incorporation into cells during 24 hours in culture. Both unpurified and purified cultures contained a small percentage of cells with labelled nuclei. All of these labelled cells were A2B5(-).

Fig.2-11. ^{3}H-thymidine autoradiography combined with immunohistochemistry of monolayer cultures after 13 days *in vitro*. Cultures were continually exposed to label in the culture medium and dipped slides were exposed 4 days before developing. Each micrograph set (a and b) are comprised of (from left to right) phase contrast, brightfield, and A2B5 immunostaining. The photographic emulsion appears as phase-dark wrinkles in the left micrographs. All black in the middle unstained brightfield micrographs represents label. Individual nuclei appear as the dense dark areas. In both (a) unpurified and (b) purified cultures ^{3}H-labelled nuclei were found only in the flat A2B5(-) glia. Most glia were labelled. No labelled nuclei were found in A2B5(+) neurons either singly or when in aggregates (arrowheads). Bar, 50μm.

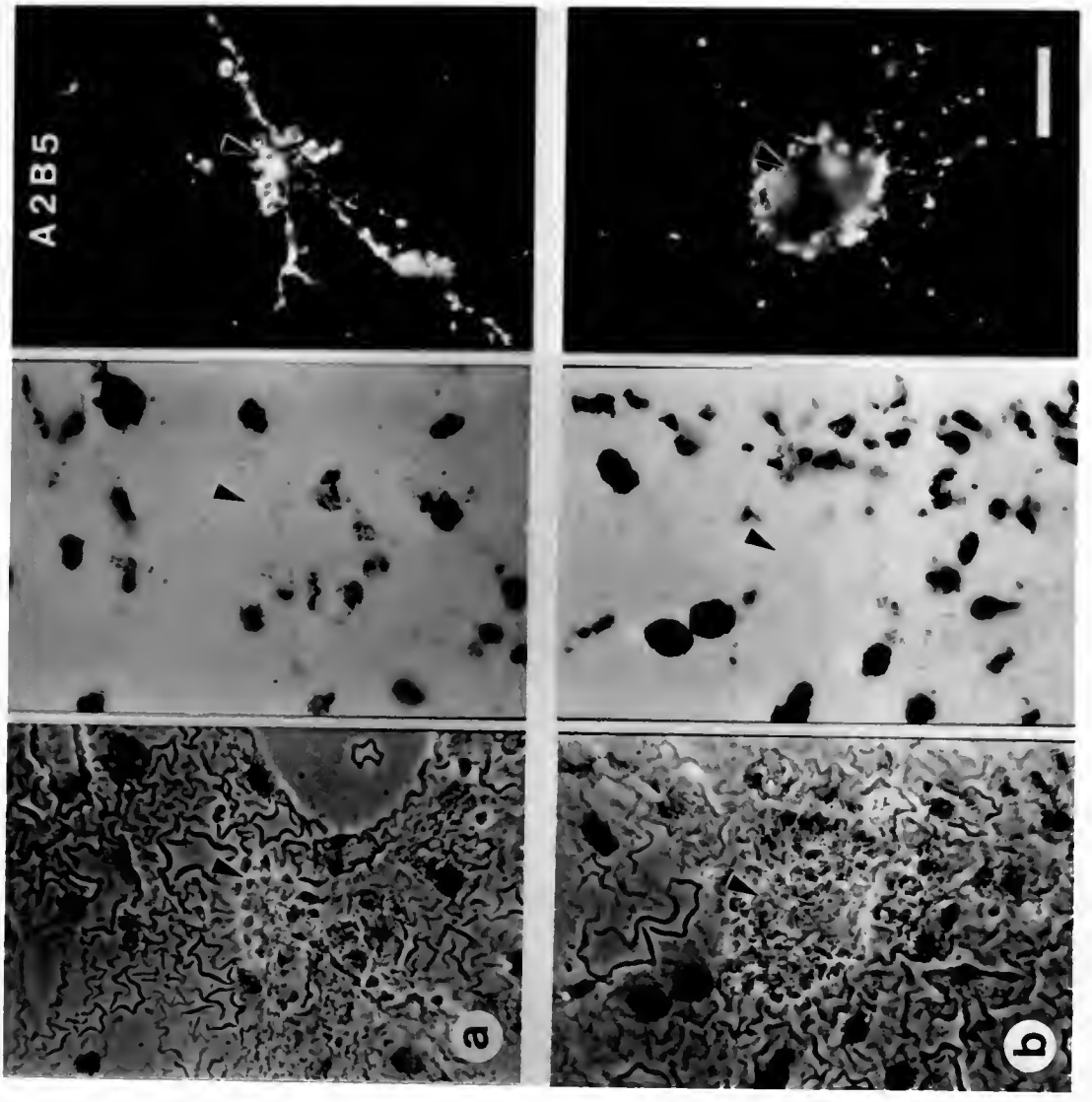
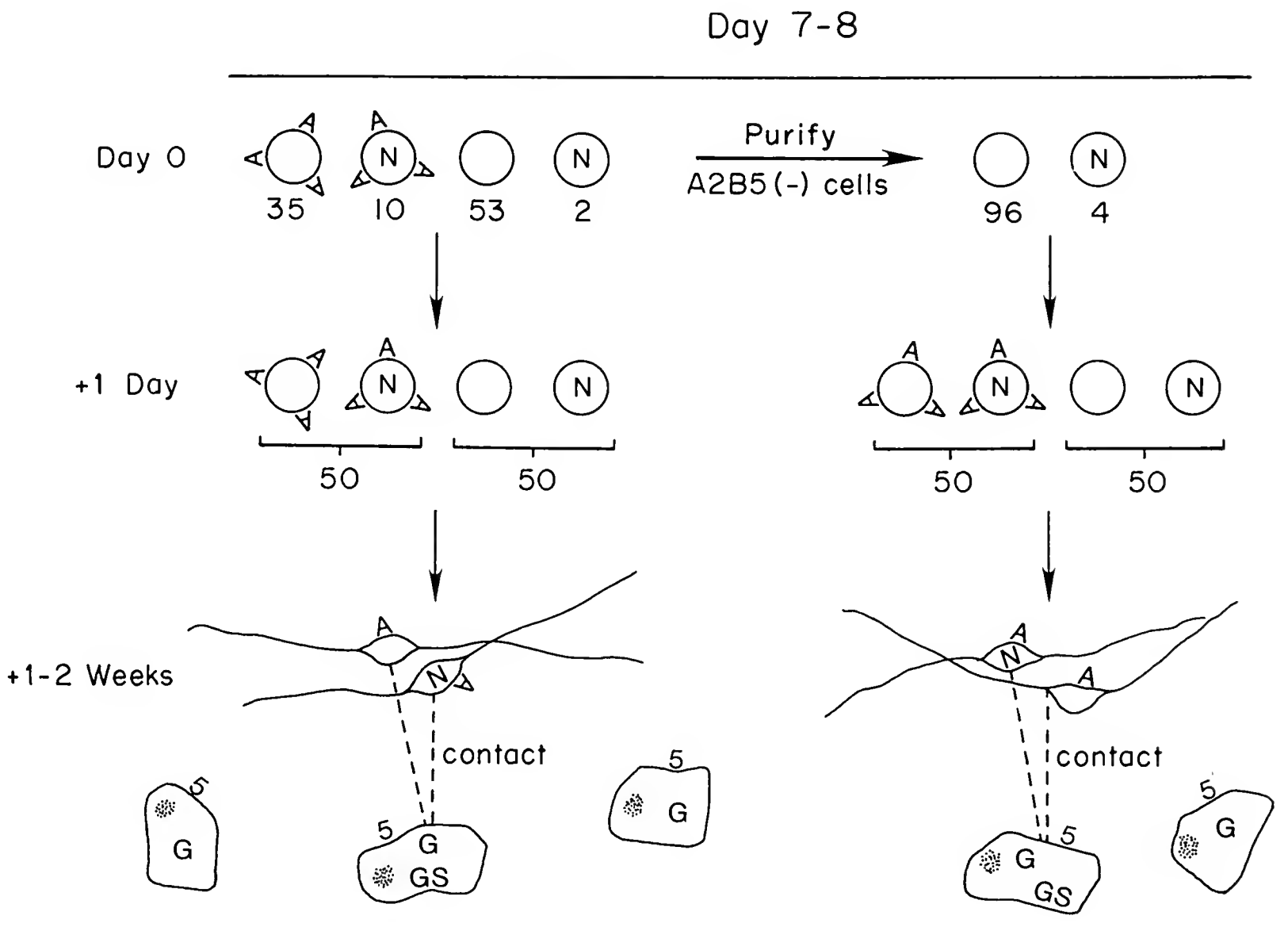

A2B5
a
b

Fig.2-12. Diagrammatic summary of the results, taking into account immuno-phenotype, morphology, and ^{3}H-thymidine incorporation of unpurified (left) and purified A2B5(-) (right) cells. Time in culture is denoted on the left. "A" on a cell indicates cell surface A2B5 antigen, "5" indicates binding of antibody 5A11, "N" indicates NF-M immunoreactivity, "G" indicates anti-glial fibrillary acidic protein immunoreactivity, and "GS" indicates anti-glutamine synthetase immunoreactivity. The numbers below cells indicate percentages of either unpurified (left) or purified (right) cells. Labelled nuclei are shown as intracellular stiplings.


Day 7-8
Day 0
35
10
53
2
Purify
A2B5(-) cells
96
4
+1 Day
50
50
50
50
+1-2 Weeks
contact
contact
G
GS
5

frame. This approach would not be possible if the tissue were retina, however, since the cells lose their competence for reaggregation and GS production in a matter of hours (Linser, 1987 and unpublished).

Separation Technique

The immunomagnetic separation technique that was used was ideally suited for my purpose. Populations of extremely pure A2B5(-) cells were obtained in a relatively short time period. The ability to separate the tectum cells based solely on surface antibody binding seemed necessary since most 7 or 8 day cells are not sufficiently differentiated to make use of other purification strategies. Immunomagnetic cell separation has been applied to neural tissues previously to purify oligodendrocyte precursors (Meier et al., 1982; Meier and Schachner, 1982) with success. This was a positive selection of the target cells using large polyacrylamide-coated beads, however, in contrast to the negative selection employed here. They reported an enrichment of oligodendrocytes from 1.5% to 91% purity and an average yield of 19%. The method described here resulted in much higher purity of cells as well as a higher yield. Also, cells could be recovered from the microspheres with a purity comparable to the oligodendrocyte selection above with much better yield.

Several major modifications of the technique used to purge bone marrow (Treleaven et al., 1984) had to be made

for optimum separations of dissociated embryonic brain cells. It was necessary to mix cells and microspheres in a medium containing DNase. This was presumably due to the release of DNA due to cell lysis. Various types of separation chambers have been used for clinical applications (Gee et al., 1987; Treleaven et al., 1984). The chamber that was used for separation was much simpler than those used for bone marrow purging. This open system proved to be entirely satisfactory and contamination due to this never occured. Lastly, satisfactory separations were obtained with a much lower microsphere/target cell ratio than is used for marrow purging. This is believed to be due to increased collisions between target cells and microspheres as a result of a higher percentage of target cells (50%) than in infected bone marrow (≈1%).

A2B5 Antigen and its Modulation

A2B5 (Eisenbarth et al., 1979) was chosen as the target cell antibody for the immunomagnetic separations. This was because of its high specificity for neurons in long-term monolayer cultures of dissociated differentiated tectum cells. Other factors that led to its choice were that it has been reported to be neuron specific in humans (Kim, 1985; Kim et al., 1986) and to bind to most or all neurons in chick brain (Schnitzer and Schachner, 1982). The antigen it recognizes is also a protease resistant ganglioside (Eisenbarth et al., 1979; Kasai and Yu, 1983).

When compared with immunostaining for neurofilaments of freshly dissociated cells it was found that a small percentage of NF(+) cells were A2B5(-). The majority of the NF(+) cells, however, were A2B5(+) (83%) and were removed. Thus the specificity of A2B5 for neurons initially is not complete and all-inclusive but encompases the majority of identifiable neurons. No other markers that react with differentiated neurons are known that react with 7 or 8 day cells. Neuron specific enolase in chick brain does not appear until later in development and apparently is not produced in brain cell cultures (Ledig et al., 1985). Aside from markers, culturing cells on neuron-selective polyornithine suggests that at least most of the A2B5(+) cells were neurons because the microsphere fraction was enriched for neurons and the purified A2B5(-) cultures did not contain them.

The appearance of A2B5 antigen on the surfaces of the purified A2B5(-) cells was curious. This phenomenon was first observed when the purified cells were incubated in A2B5 and then the fluorescent secondary antibody. This was done for fear that some A2B5(+) cells may have quickly cleared the monoclonal from their surfaces after the separation. Subsequent experiments revealed that no reduction in the number of A2B5(+) cells occured after a day in culture (data not shown). Therefore, the correct assay to assess the degree of depletion of A2B5(+) cells

was to incubate the purified cells in only the secondary antibody since rapid turnover of the antigen did not seem to occur.

As was stated earlier, the antigen recognized by A2B5 is a ganglioside (Eisenbarth et al., 1979; Kasai and Yu, 1983). These sialoglycosphingolipids are believed to be synthesized in an progressive fashion from individual sugars transfered from nucleotide conjugates (Ledeen, 1985). This apparently takes place in the Golgi apparatus and probably the smooth endoplasmic reticulum through membrane bound multienzyme complexes specific for synthesis of each ganglioside. This occurs only in the cell soma of neurons in the chick visual system (Landa et al., 1979) after which the gangliosides translocate to nerve endings via fast axonal transport (Ledeen, 1985). In fractionated cells gangliosides are found predominantly in the synaptosomal and microsomal fractions (Hamberger and Svennerholm, 1971). Gangliosides exhibit turnover and are degraded primarily in lysosomes in an ordered fashion and evidence suggests that postsynthetic processing of them does not occur (on the cell surface) between synthesis and degradation (Ledeen, 1985).

The mechanisms that control the synthesis and export of gangliosides to the cell surface not at nerve endings are not well understood. From what is known about ganglioside biosynthesis (Ledeen, 1985) it is probable that

the appearance of A2B5 antigen on purified cell surfaces is not due to the modification of existing gangliosides on the surface or even intracellularly. This cannot be ruled out, however, because increases in the complex gangliosides (including G_{Q1c}) during development of the chick optic tectum are correlated with decreases in simpler precursors (G_{D3}) (Rosner, 1980). Other possibilities that may explain the appearance of A2B5 antigen are new synthesis and export to the surface, or export of an intracellular pool. Since it is known that chick brain cells have intracellular pools of A2B5 antigen (see below; Schnitzer and Schachner, 1982) this possibility is quite real. Export to the surface could be via exocytotic vesicles or via a ganglioside transfer protein found in brain (Gammon and Ledeen, 1985). These types of export mechanisms could account for the appearance of A2B5 antigen on the surfaces of purified cells within the several hours that it has been seen to occur (see Results).

The number of "recruited" A2B5(+) cells was a function of the number of original A2B5(+) cells that were removed as demonstrated by the calculated incomplete separations. The question remains as to whether this triggered modulation of A2B5 antigen was a result of the removal of neurons or of A2B5(+) cells. Some (20%) of the A2B5(+) cells that were removed are known to be neurons because they contained neurofilaments. The remaining 80% were of

unknown type. They may, however, have been neurons that either did not contain neurofilaments because of lack of synthesis in the tissue or because of severing of axons during dissociations. The results of culturing cells recovered from the microspheres on glass and polyornithine suggest that these are neurons because cultures from these A2B5(+)-enriched cells appeared to be neuron-enriched. Later on, there was a tight correlation between a cell being A2B5(+) and having a neuronal morphology in long-term monolayer cultures. Therefore, modulation of A2B5 antigen on purified A2B5(-) cell surfaces may be a response to neuronal depletion as effected by the removal of A2B5(+) cells. Consistent with this hypothesis, surface A2B5 antigen modulation has been shown to occur with mouse cerebellar astrocytes in culture in response to complement-mediated depletion of neurons using an independent neuronal marker (Nagata et al., 1986).

The inability to prevent recruitment of new A2B5(+) cells by mixing back the cells that were removed implies that the recruitment was very rapid and irreversible. There exists the possibility that this was due to damage of the cells that were removed from the microspheres by the second trypsinization. This seems unlikely, however, since the cells were trypsinized before dissociation. These recovered cells also grew well in culture. It appears that the events that led to the expression of A2B5 antigen on the

recruited cells were irreversible within the time frame in which they were remixed (an hour).

A main question that remains is the significance of A2B5 antigen and its appearance on the surfaces of purified cells. Gangliosides are major constituents of the glycocalyx of neural cells (Ledeen, 1985). Their general stability in the membrane makes them ideal candidates for roles such as adhesion and recognition. Gangliosides are known to be the neural cell surface receptors for tetanus toxin (G_{D1b} and G_{T1b}) and for cholera toxin (G_{M1}) (Ledeen, 1985). Gangliosides are also thought to influence in some way the formation of synapses (Grunwald et al., 1985) and the process of myelination (Ledeen, 1985). In culture, purified gangliosides have been shown to mediate adhesion of embryonic chick retina cells (Blackburn et al., 1986) and to alter the morphology and growth of astrocytes from fetal rat brain (Hefti et al., 1985). It is possible that the ganglioside recognized by A2B5 on cell surfaces similarly may function as a recognition molecule. It is unlikely that it serves as an adhesion molecule since cells that had bound antibody on their surfaces did not appear to have diminished ability to form either heterotypic or homotypic contacts with other cells. It may even be proposed that A2B5 antigen is a molecule that is involved in the communication between neurons and other cells since A2B5 is neuron-specific in monolayer cultures, here, and

since its modulated appearance on cell surfaces depends on the removal of cells already expressing it at their surface.

Development of Purified Cells

It is clear that A2B5(+) cells were recruited in purified cultures. It is also clear that neurons appeared in purified cultures when grown on a nonselective substratum. Initially, the small proportion of identifiable neurons present before separation were depleted by 83% as evident by NF immunoreactivity. Degeneration of purified cells on polyornithine also suggests that neurons were depleted because no cells survived. Presumably, they would have grown if they were present in the purified cells. This degeneration occured even though the cells had become A2B5(+). Thus, the presence of A2B5 antigen on a cell surface does not in itself correlate with survival on polyornithine. Microsphere fraction cells (A2B5(+)-enriched), on the other hand, resulted in the visually most dense network of neurons, which suggests that the majority of neurons were A2B5(+).

When grown on a nonselective substratum such as glass for ≈ 1-2 weeks there appeared to be no decrease in the density of A2B5(+) neurons or of neurofilaments in purified cultures as compared to unpurified monolayers. If the purified A2B5(-) cells were initially devoid of the majority of neurons as is suggested above, then, neurons

must have come from preexisting nonneuronal cells to exhibit the same density as in unpurified cultures (see ^{3}H-thymidine discussion below). The simplest explanation for equal densities of neurons is that A2B5 expression on the surfaces of cells is irrelevant to the development of neurons in long-term monolayer cultures. A2B5 antigen may have been modulated up and down on cell surfaces in both unpurified and purified cultures. Then, the A2B5(+) neurons at the culture endpoints may not have developed from the A2B5(+) cells seen initially or after a day in culture. The phenomenon of recruitment of A2B5(+) cells in purified cultures may be separate from the appearance of neurons in these cultures. No experiments were performed that could conclusively demonstrate which of the possibilities had occured.

On the other hand, neurons as defined by morphology and/ or neurofilament content were almost always surface A2B5(+) (>90%) in long-term monolayer cultures. Conversely, cells that expressed A2B5 antigen on their surfaces always had a neuronal morphology in monolayer cultures. Additionally, the presence of cells that were surface A2B5(+) always preceded the development of neurons in long-term cultures. A2B5(+) cells were present at the outset in unpurified cultures, and were present after one day in purified cultures. These correlations raise the possibility that A2B5(+) neurons in long-term cultures developed from

the A2B5(+) cells that were seen after one day in culture. The A2B5(+) neurons that developed in unpurified cultures may have originated from the A2B5(+) cells that were present when the tissue was dissociated (which were presumably were the same cells that were A2B5(+) after a day in culture). The fact that either no change or a slight increase in the percentage of A2B5(+) cells occured in unpurified cells after one day in culture is consistent with A2B5 antigen not being modulated in unpurified cultures. Similarly, the A2B5(+) neurons that developed in purified cultures may have originated from the recruited A2B5(+) cells seen after a day in culture. If this hypothesis were true, then, this would imply the existence of a previously unidentified intermediate cell type in the optic tectum neuronal lineage. This cell would have the characteristics of being A2B5(+)/ NF-M(-) and would be susceptible to degeneration on polyornithine.

The analysis of GS in culture revealed another manner in which purified cultures were identical to unpurified cultures. GS was produced in purified monolayer cultures in an indistinguishable pattern from unpurified cultures. Quantitatively, the expression of GS in the two types of cultures was also the same. These results suggest several possible explanations. One is that the purification did not result in separation of neurons and glia. This has been discussed above. Another is that a small number of neurons

may be able to induce production of GS in a certain number of glia as well as a large number of neurons could. This possibility is compounded by the fact that there are many different types of neurons in the optic tectum (LaVail and Cowan, 1971a) and it is not known which types are capable of GS induction. Maybe the small percentage of A2B5(-) neurons that were in the purified cells were the neurons that induced GS. Another possible explanation is that neurons were recruited in the neuron-depleted purified cells in a rapid manner so that they could interact with the glia to produce GS. This would have to have been within about a day as was determined by the isolation of cells in methyl cellulose. If the appearance of A2B5 antigen on cell surfaces was an indication of commitment to becoming a neuron as is suggested then the ability to induce GS may also have occured rapidly as did the expression of A2B5 antigen. However, this hypothetical change was not sufficient to ensure survival of cells on polyornithine.

It is also worth mentioning that levels of GS produced in aggregate cultures made from cells that were isolated in methylcellulose were identical to levels produced by immeadiately reaggregated dissociated cells. This is surprising since it was shown that a much greater number of A2B5(+) cells existed initially in cells from the methylcellulose (≈75%) than in immeadiately reaggregated cells (≈50%). So either the number of A2B5(+) cells has

nothing to do with the amount of GS produced or the proper ratio of neurons to glia is somehow obtained. Although it was shown that GS is produced in glia under clusters of A2B5(+) neurons it could not have been determined whether more GS activity was induced under large clusters than under small clusters. The proper ratio of neurons to glia could have been accomplished by division of the glia since these cells have been shown to incorporate ^{3}H-thymidine in culture.

There appears to be an absolute correlation between having cell surface A2B5 antigen and not synthesizing new DNA in cultures of tectum cells. The lack of ^{3}H-thymidine labelled nuclei in neurons in cultures from unpurified day 7 or 8 cells was expected since other workers have found that the majority of neurogenesis in the tissue has already occured by this time (LaVail and Cowan, 1971b; Puelles and Bendala, 1978). The fact that no labelled neurons were found suggests that all of the neurons that survived in culture had completed their terminal S-phase by day 7. Similarly, the lack of any labelled nuclei in recruited A2B5(+) cells that appeared in purified cultures demonstrated that new DNA synthesis was not required for cell surface expression of A2B5 antigen. The antigen may have been expressed via a mechanism as discussed in the section above. The neurons that developed in these cultures, likewise, did not require new DNA synthesis for

differentiation (i.e. the synthesis of neurofilaments). This finding suggests that these neurons originated from cells that were already born. This may have been from a resting blast cell that was limited to the choices of either differentiating into a neuron or dying. Alternatively, these neurons may have originated from cells that would have become glia had the need for more neurons not occured. This possibility would be exciting, since it suggests that a change in phenotype or even cell type may occur in post-mitotic cells if the neurons that developed in purified cultures were recruited as is suggested.

Glial Development and Plasticity

Perhaps the most interesting question that the results here pose is: From what population of cells were the recruited neurons taken? Two theoretical possibilities exist. The recruited cells either would have become something else had there been no depletion, or they were resting blast cells that normally would degenerate if not needed. And since these cells were of neuroectodermal origin, if they were not to become neurons then they were to become glia. The results presented here do not support one or the other possibility. Analysis of cell lineage in the rat retina by using recombinant retroviral vectors has shown that both neurons and glia are produced from common progenitors throughtout development (Turner and Cepko, 1987). However, results with purified cells from day 12 or

13 optic tectum suggest that the recruited cells in cultures stem from the astrocyte lineage (Chapter 3). The majority of the recruited A2B5(+) cells in these purified cultures showed immunoreactivity for GS thus identifying them as glia. There is also evidence in the chick peripheral nervous system that certain glial precursors are capable of being diverted to a neuronal lineage under certain transplantation conditions (Le Lievre et al., 1980).

The mechanism of recruitment is also unclear. One possibility is that there exists either a negative feedback system between A2B5(+) and A2B5(-) cells or a positive feedback system between A2B5(-) cells only. With the negative feedback system the A2B5(-) cells would have recognized the loss of the A2B5(+) cells by some mechanism and then reacted as a result of this. With the positive feedback system the A2B5(-) cells would have sensed an increase in the density of A2B5(-) cells in the purified cultures and reacted to replenish A2B5(+) cells. The cell isolation experiments, however, clearly rule out the latter possibility since recruitment of A2B5(+) cells occured as a result of a loss of contact between all cells. What remains in question then is whether the communication between A2B5(+) and A2B5(-) cells is via cell contact or soluble factors.

An interesting finding concerning glial development in culture is the lack of appearance of oligodendrocytes in culture. These results using an antibody for the marker galactocerebroside (GC) essentially confirm the results of Linser and Perkins (1987a) who failed to find cells positive for the oligodendrocyte markers myelin basic protein and S-100 (Linser, 1985). This is in contrast to cultures made from day 12 or 13 tectum cells where GC(+) cells are present and develop in vitro (Chapter III). Thus it seems that either future cellular interactions were disrupted that were required for oligodendrocytes to develop, or that the culture conditions did not contain some growth factor(s) that was required earlier in development. It should be noted that these results are in contrast to those with rat brain cells where oligodendrocytes appear in cultures that do not contain them initially (embryonic day 10) on time with those that appear in vivo 13-14 days later (Abney, Bartlett, and Raff, 1981).

CHAPTER III
SEPARATION OF DAY 12-13 CELLS

Introduction

The previous chapter described an immunomagnetic separation method to separate cells from early (day 7-8) embryonic chick optic tectum. This method resulted in extremely pure populations of cells that were negative for the cell surface marker A2B5 (Eisenbarth et al., 1979). It was found, however, that the A2B5 antigen was modulated on the surfaces of about half of the purified cells in direct response to the depletion of A2B5(+) cells. Neurons were also apparently recruited in these cultures. Thus, day 7 and 8 optic tectum cells showed a remarkable ability to maintain the correct number of A2B5(+) cells and neurons. It was not clear, though, from what population of cells the recruitment was occuring. This was largely due to the fact that no glial differentiation markers which occur later in development were present at days 7 and 8 to identify definitive glia.

During development of the chick optic tectum, commonly recognized glial differentiation markers do not appear until relatively late in development (Linser and Perkins, 1987a). Glutamine synthetase (GS) is detectable by immunohistochemistry in some astrocytes at day 9 and is

produced eventually in most if not all astrocytes. Glial fibrillary acidic protein (GFAP) appears in a small population of astrocytes beginning at day 16 of development. The oligodendrocyte specific marker myelin basic protein (MBP) appears on day 12 followed by S-100 on day 16. This is in striking contrast to the neuronal marker, neurofilaments, which appears beginning on day 3 of development when neurons begin to exit the mitotic cycle (Bennett and DiLullo, 1985). Unfortunately, however, another widely used neuronal marker, neuron-specific enolase, does not appear in chick brain until much later (day 17) (Ledig et al., 1985).

The monoclonal antibody A2B5 which was used to tentatively identify and remove neurons immunomagnetically seemed at first to be a relatively stable marker with freshly dissociated cells. It reacted with approximately 50% of dissociated cells from days 7-13. If it was assumed that A2B5 was reacting with the same population of cells throughout this range of ages, it would be interesting to examine whether induced deficiencies of A2B5(+) cells and/or neurons would be compensated for if day 12 or 13 A2B5(-) cells were purified as they were with day 7 and 8 cells. Since two known glial differentiation markers have appeared in the tissue by this time (GS and GC), it might be hypothesized that the capacity for neuronal "recruitment" from other cells would be very limited. In several other

respects, as stated by LaVail and Cowan (1971a), age 12 tissue differs from earlier ages as follows: By this time all mitosis in the neuroepithelium has ceased (Cowan et al., 1968). The major 6 laminations of the tectum have been arranged, and many cells have obtained their relative final positions. Retinal axons by now have penetrated all parts of the tectum, and by this age the superficial tectal laminae are dependent upon retinal contact for survival (Kelly and Cowan, 1972).

Therefore, I have immunomagnetically purified A2B5(-) cells from day 12 and 13 cells for purposes of studying their development <u>in vitro</u> as compared to unpurified cells. A2B5 was compared to the differentiation markers present at this time (NF and GS) as well as to the oligodendrocyte marker galactocerebroside (GC) in freshly dissociated cells. Monolayer cultures of purified and unpurified cells were analyzed by immunohistochemistry for these markers and for glial filaments (GFAP). ^{3}H-thymidine autoradiography was also performed on cells and monolayers to examine the role of new DNA synthesis in recruitment and differentiation. Reaggregation of cells to elicit GS production as an indicator of neuronal-glial interaction was not performed, since this age tissue is beyond the age at which this can be done successfully (unpublished). Similarly, the effects of polyornithine substrata on glial degeneration (Adler et al., 1979) could not be utilized,

because glia can survive on this substratum by this age (see Results).

I have found that glia which have been deprived of neuronal contact can alter their immuno-phenotype drastically. Day 12 and 13 tectum cells have a very limited ability, if any, to replenish depleted neurons *in vitro*. Instead, phenotypes were found in purified cultures that were intermediate between, or showed characteristics of both, neurons and glia. These were not found in unpurified cultures and apparently represented reactions to depletion of neurons. Taken with previous findings in Chapter II, the inability to replenish neurons coincident with the appearance of abnormal phenotypes suggest that the non-neuronal cells in day 12 and 13 tissue (glia) are restricted in their potential to become neurons *in vitro*.

Materials and Methods

White Leghorn chick embryos were used throughout this investigation. Fertile eggs were purchased from the Division of Poultry Science, University of Florida, and stored at 15°C until initiation of incubation at 37.5°C in a humidified egg incubator. For this series of investigations, 12 and 13 day embryonic optic tecta were dissected as described in Chapter II.

To obtain sufficient quantities of dissociated cells treatment of tissue with two different proteases was required. Tissue was minced finely and then incubated in

5mg/ml neutral protease (Dispase; Boehringer Mannheim Biochemicals, Indiannapolis, IN) for 1 hour with aggitation followed by incubation in 0.4% Trypsin (Nutritional Biochemicals, Cleveland, Ohio) for 30 min., both at 37°C. Dissociation of tissue into single cells was as described in Chapter II.

Adherent monolayer cultures were prepared on glass or polyornithine as described in the previous chapter. Briefly, 10^6 cells/ well were plated on coverslips in 24 well tissue culture plates in Medium 199 supplemented with 10% fetal bovine serum. Monolayer cultures were kept in a standard tissue culture incubator in a 5% CO_2/ air atmosphere. Cultures were fed with fresh medium approximately every other day.

Cell "isolation" in methylcellulose was accomplished by suspending dissociated cells in a semisolid medium of 1.3% methylcellulose in Medium 199 according to the previous chapter.

Immunomagnetic Separations

Immunomagnetic separations were carried out exactly as described previously and so will not be described further here. Cells assessed for cell surface A2B5 immunofluorescently following a separation were fixed with 1% formaldehyde in phosphate-buffered saline (PBS) for 30 min., rinsed, then incubated for 30 min. in a 1/50 dilution of fluorescein-goat anti-mouse IgM (FITC-GAM; Boehringer

Mannheim) in PBS with 5% normal goat serum added. Cells assayed after one day in culture were incubated live in 1/25 A2B5-conditioned hybridoma medium + 10% heat-inactivated fetal bovine serum for 30 min. on ice and then processed as above. Plating efficiencies of cells were determined retrospectively from Ektachrome slides by counting the number of cells that adhered to the polyornithine coated coverslips initially after the experiment and after 1 day in culture. No attempt was made to determine cell numbers or densities in longer-term cultures.

Immunoradiometric Assay for A2B5

An immunoradiometric assay (Hunter, 1978) was used to quantitate cell surface binding of A2B5 both immediately after the separation and after 1 day in culture. For this, ^{125}I-labelled antibodies were prepared by the Iodo-Gen method (Pierce Chemical Co., Rockford, IL) according to the manufacturer's directions using polyclonal goat anti-mouse IgG +IgM + IgA (Organon Teknika- Cappell, West Chester, PA). Cells assayed for cell surface A2B5 were processed exactly as those for the immunofluorescent assay, with the iodinated secondary antibody in place of the fluorescent secondary antibody. Processed coverslips were placed in vials and bound radioactivity was measured with a Beckman Gamma 7000 (Beckman Instruments, Norcross, GA). Specific DPMs were obtained by subtracting mean nonspecific DPMs

from total mean DPMs of triplicate or quadruplicate coverslips. Nonspecific background DPMs were obtained by incubating unpurified cells in an irrelevant monoclonal antibody specific for pipefish vitelline envelope (provided by Dr. P.C.Begovac, Whitney Laboratory) for 30 min. on ice and then processing them identically as those incubated in A2B5.

Immunohistochemistry

All immunohistochemistry of cells and monolayer cultures was performed as described in the previous chapter except as noted below. 5A11 (Linser and Perkins, 1987b) immunohistochemistry of monolayers was carried out identically to that for A2B5. Immunostaining of cells for glutamine synthetase was accomplished by permeablization after formaldehyde fixing with 95% ethanol for 2 min. at -20°C, rinsing, and incubation in a 1/100 dilution of polyclonal anti-GS (Linser and Moscona, 1979) for 30 min. Other NF antibodies rasied against rat neurofilaments and a monoclonal antibody specific for GFAP (DA3, NN18, anti-MSH, A5; kindly provided by Dr. G. Shaw, Univ. of Florida) were also used to immunostain monolayers.

^{3}H-Thymidine Incorporation

New DNA synthesis in cell cultures was investigated by ^{3}H-thymidine autoradiography combined with immunohistochemistry as described in the previous chapter.

Results

The dissociation protocol used for younger tissue (Chapter II) did not produce satisfactory numbers of single cells when using older tissue (day 12 and 13). Therefore, the double protease treatment was adopted. This still resulted in only approximately $5x10^6$ cells/ embryo. The double protease treatment was used only because a greater number of viable cells could be obtained for experiments and the results herein are not believed to reflect an effect caused by the tandem proteases (see Discussion).

Markers for Dissociated Cells

Unless otherwise specifically stated, the reference as to whether a cell is A2B5(+) or (-) refers to cell surface binding of this antibody only. With dissociated day 12 and 13 optic tectum cells, discrete populations were labelled by antibodies against two recognized glial antigens and one neuronal antigen, as well as with A2B5 (Figs.3-1,3-2). Approximately 10% of dissociated cells were reactive with the polyclonal antibody against neurofilaments. The majority of these were A2B5(+). It was more difficult to attempt to quantitate this because, unlike younger cells, there was an absence of reactive filaments in the cell cytoplasm around the nucleus. NF-M(+) (Bennett et al., 1984) cells were less distinct and mostly showed immunoreactivity in what was apparently membrane blebs of

Fig.3-1. Graph comparing the cell surface reactivity of marker A2B5 to reactivity against NF, GS, and GC. The percent of total dissociated cells that react with each antibody is quantitated on the abscissa. The percent that react with purified cells is obtained by multiplying the percents on the A2B5(-) side by 2. A2B5 bound to approximately 50% of dissociated cells as shown on the top. Anti-NF reactivity was present in ≈12% of the cells and 10 out of 12 of these were A2B5(+) (this data only is from days 7 and 8 cells; see text). Anti-GS antibodies reacted with ≈40% of total cells and all of these were A2B5(-). Similarly, anti-GC antibodies reacted with only A2B5(-) cells (6%) and therefore were enriched in the purified fraction. Approximately 95% of the purified cells could be identified as reactive with the markers NF, GS, and GC. More than 90% of the purified cells could be identified as either GS(+) astrocytes or GC(+) oligodendrocytes.

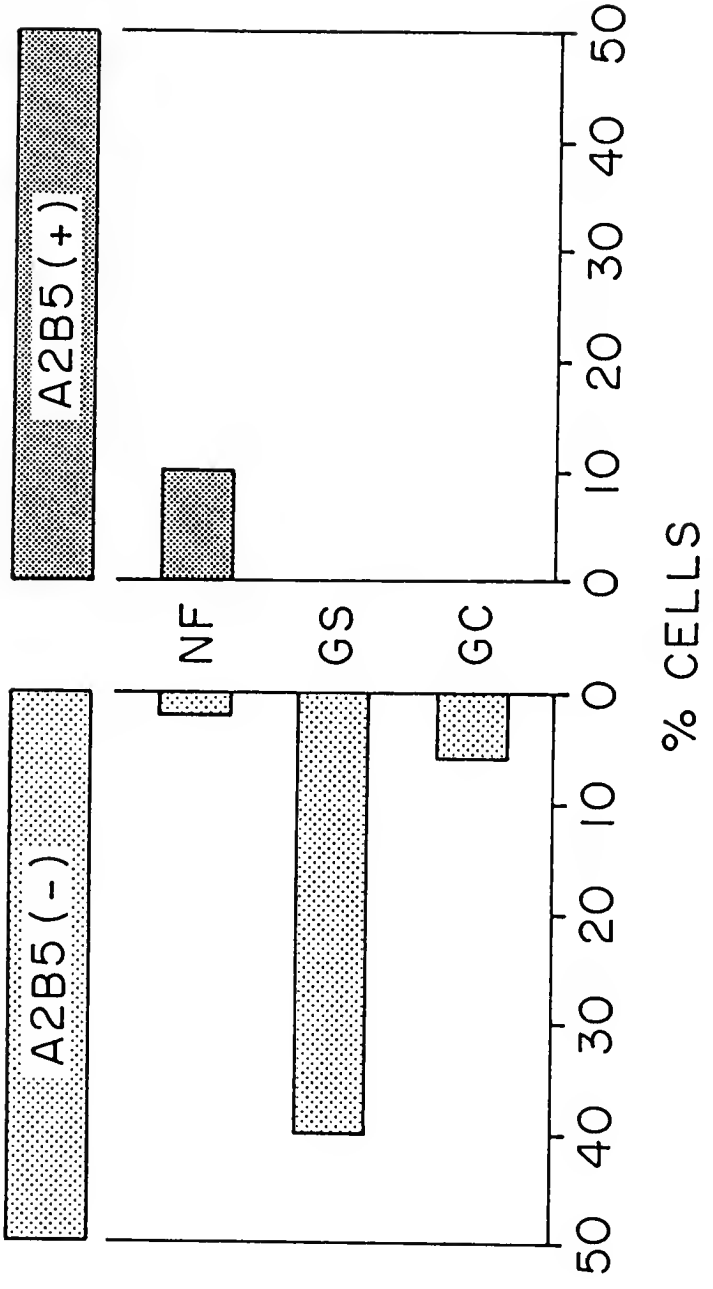
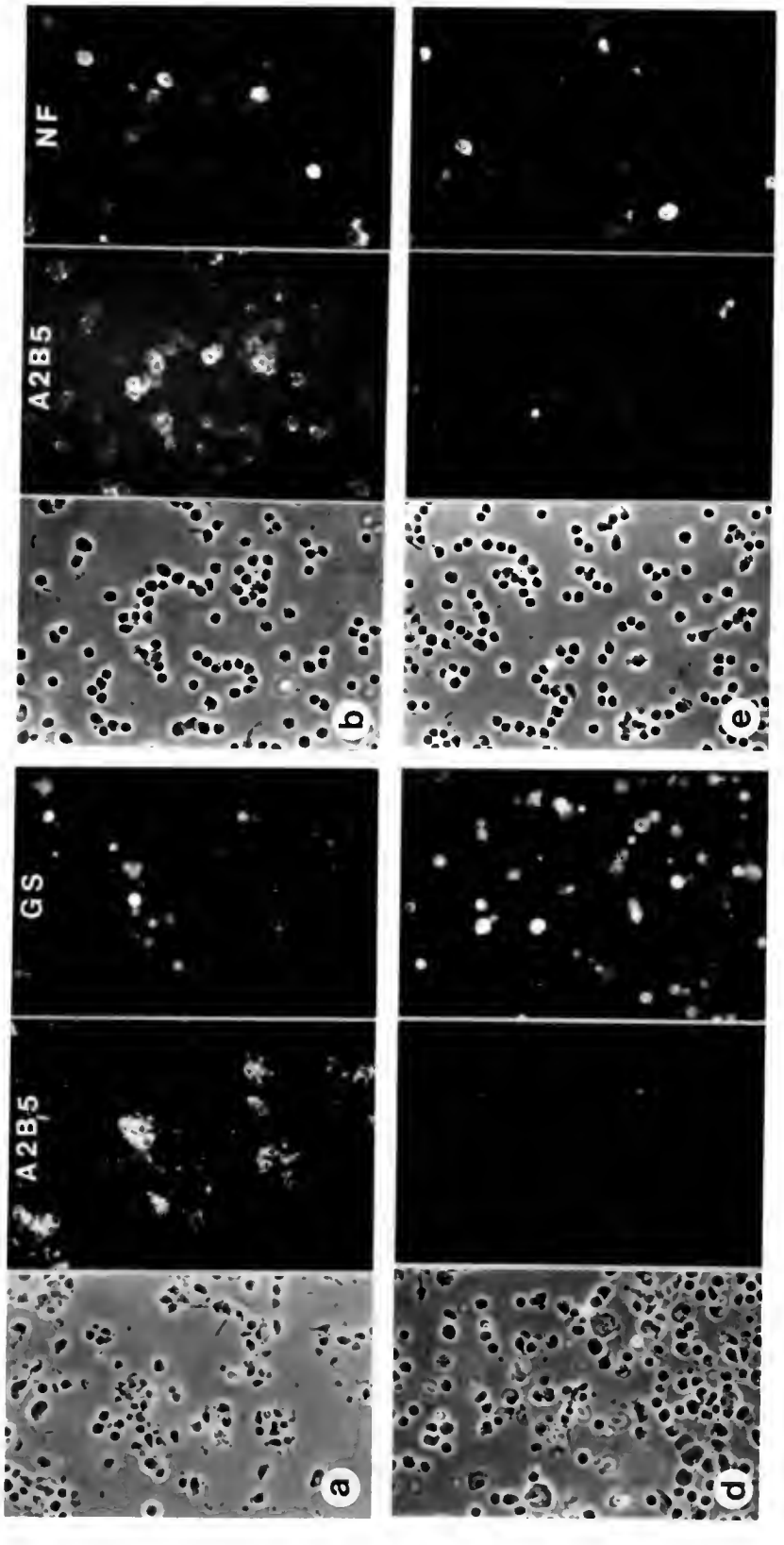

A2B5(+)
A2B5(-)
NF
GS
GC
0
10
20
30
40
50
% CELLS

Fig.3-2. Double-label immunostaining of dissociated unpurified (a-c) and purified (d-f) cells with A2B5 and for either GS (a,d), NF (b,e), or GC (c,f). These micrographs correspond to the graph shown in Fig.2-1. Refer to that figure for quantitative analysis of double labelling. Bar, 50μm.


A2B5
GS
A2B5
NF
a
b
d
e

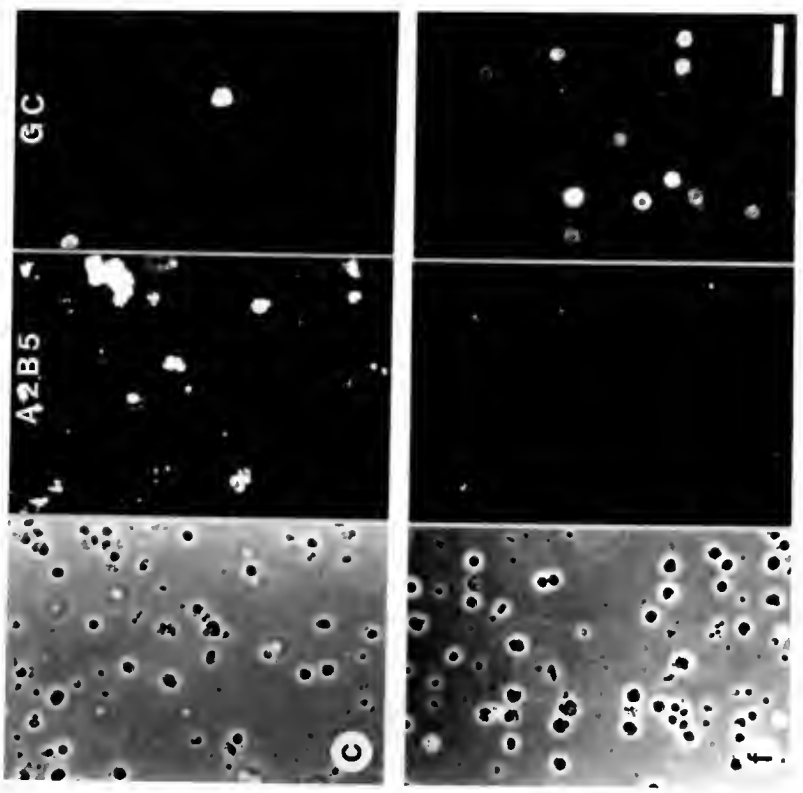


Fig. 3-2 Cont'd.

retracted or severed axons on the surfaces of the cells. Therefore, the most accurate count of NF-M(+) cells was obtained with the day 7 and 8 cells described in the previous chapter. Even this data may be an underestimate of the number of NF-M(+) cells for the above reasons.

Polyclonal antibodies specific for the definitive glial marker GS reacted with approximately 40% of dissociated day 12 and 13 cells. These cells were A2B5(-) and so the purified population of A2B5(-) cells (see analysis of purification below) was approximately 80% GS(+) (Figs.3-1,3-2). Thus, the astrocytes that were identifiable as such by immunoreactivity with anti-GS were concentrated in the purified population.

Dissociated cells at this time also were reactive with a monoclonal antibody specific for the oligodendrocyte marker galactocerebroside (GC; Ranscht et al., 1982). Approximately 6% of dissociated cells were GC(+). Similar to the analysis of GS, all of these cells were A2B5(-) and so the purified A2B5(-) cells were 12% GC(+) (Figs.3-1,3-2). Taken with the results for GS, >90% of the purified cells could be identified as glia by these two differentiation markers. A small population of the purified cells (≈4%) could be identified as neurons that contained neurofilaments.

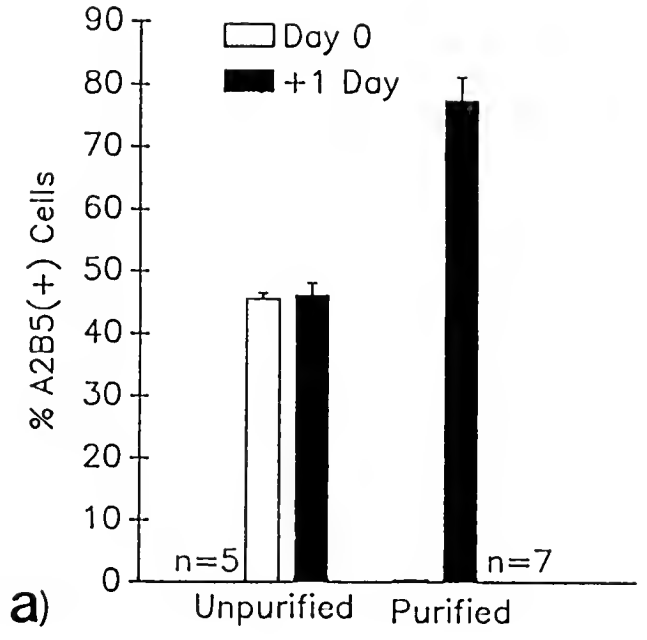
Effects of Substrata

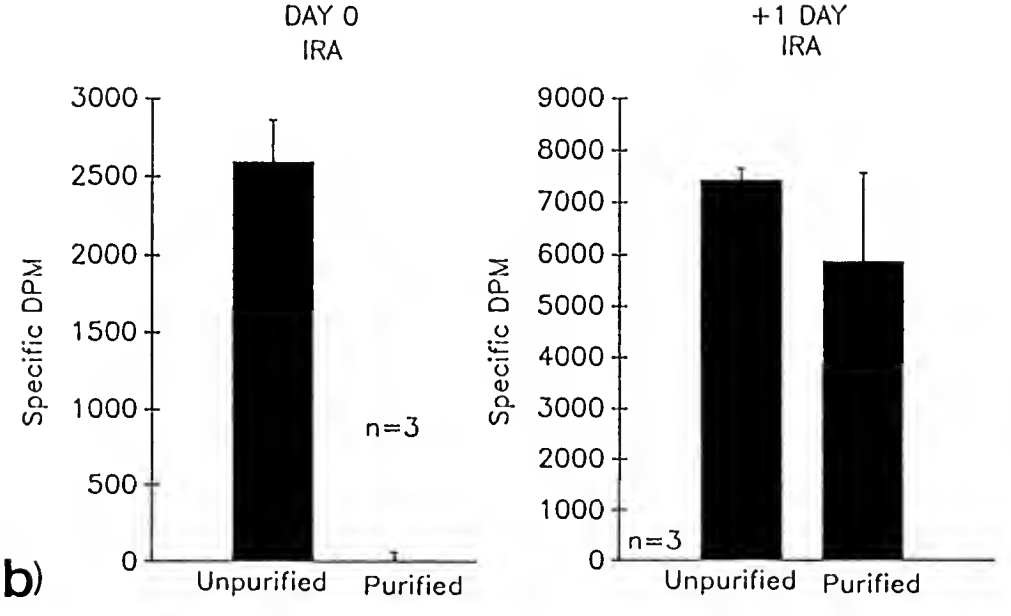
As in the preceeding chapter, both unpurified and purified A2B5(-) cells were cultured on substrata of either glass or polyornithine. It was found that although there were slight differences in the development of monolayers with regards to the extent of cell aggregation before spreading, polyornithine was not found to be selective for neurons as it was with day 7 and 8 cells. No cell phenotype that was observed to develop on glass was absent from monolayers on polyornithine. Thus, the degeneration of glial precursors on polyornithine was not manifested in day 12 and 13 cells.

A2B5 Antigen Modulation

By the immunofluorescent assay described here and in the preceedind chapter, the immunomagnetic separations resulted in extremely purified populations of A2B5(-) cells. Unpurified cells were approximately 45% A2B5(+) (Fig.3-3). Purified cells were virtually free of A2B5(+) cells, with a typical purity of >99.99% A2B5(-). After one day *in vitro*, however, large numbers of A2B5(+) cells (≈80%) were present in the cultures of initially purified cells (Fig.3-3). No increase in A2B5(+) cells was observed in cultures made from unpurified cells. Thus, A2B5 antigen is apparently modulated on the surfaces of the majority of A2B5(-) cells in purified cultures, but not on the surfaces of A2B5(-) cells in unpurified cultures. Plating

Fig.3-3. Analysis of initial purification of A2B5(-) cells and A2B5 antigen modulation on purified cell surfaces by immunofluorescence (a) and immunoradiometric (b) assays. The immunofluorescent assay (a) indicated that the purified population of cells was initially devoid of A2B5(+) cells. After 24 hours in culture, however, the purified population was ≈80% A2B5(+). The unpurified population did not show any increase in A2B5(+) cells. The immunoradiometric assays (b) confirmed that the purified cells were devoid of cell surface A2B5 antigen initially (left graph). After one day in culture the purified cells expressed levels of A2B5 antigen approaching those of unpurified cells (right graph). Shown in all graphs are means ± s.e.m. DPMs are higher in the +1 day immunoradiometric assay due to use of ^{125}I-labelled antibody with 3x the specific activity.


90
80
70
60
50
40
30
20
10
0
% A2B5(+) Cells
Day 0
+1 Day
n=5
n=7
Unpurified
Purified
a)


DAY 0
IRA
+1 DAY
IRA
3000
2500
2000
1500
1000
500
0
Specific DPM
n=3
Unpurified
Purified
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
Specific DPM
n=3
Unpurified
Purified
b)

efficiencies of unpurified and purified cells after a day in culture were nearly identical and ranged from approximately 60-90% as determined by retrospective counts from slides.

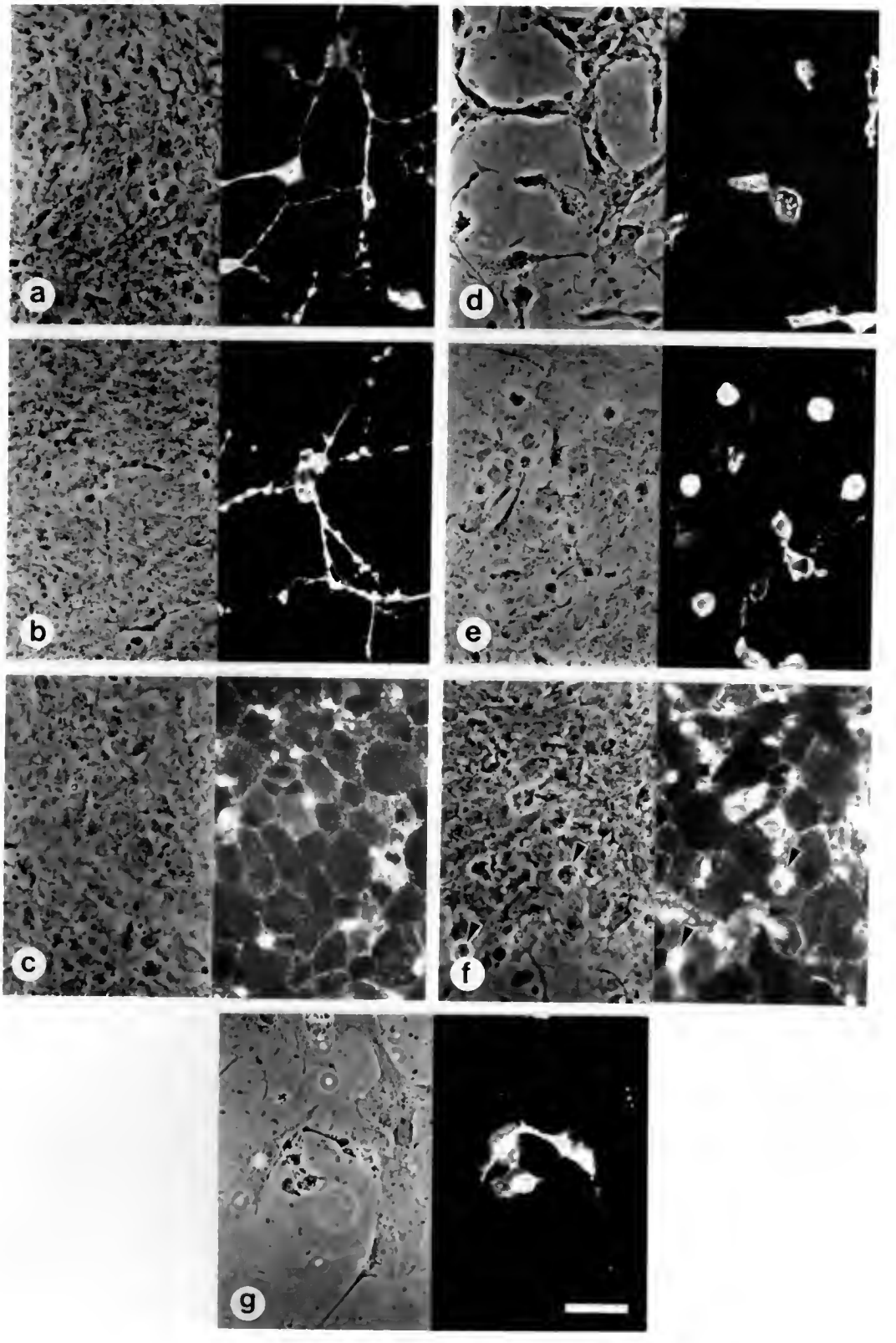
To be certain that the observation of the modulation of A2B5 antigen by immunofluorescence was accurate, immunoradiometric assays were performed to quantitate cell surface A2B5 antigen. Assays identical to the immunofluorescent ones were performed using an iodinated secondary antibody. The results of this assay are shown in Fig.3-3 and confirm the immunofluorescent results of depletion of A2B5(+) cells in purified populations. This assay also established that the immunofluorescent assay was a sensitive and satisfactory method for assessing the presence or absence of A2B5(+) cells. Similarly, the immunoradiometric assay confirmed the presence of near control levels of A2B5 antigen on the surfaces of purified cells after one day <u>in vitro</u> (Fig.3-3). There was no quantitative increase in the levels of cell surface A2B5 antigen in unpurified cultures after one day.

Similar to the results with day 7 and 8 cells, an increase in the number of A2B5(+) cells (≈75%) was observed after dissociated cells were kept suspended in isolalation in a semisolid medium containing methylcellulose for 24 hours.

Development of A2B5(+) Cells In Vitro

Although up to this point the phenomenon of appearance of A2B5(+) cells in purified cultures of day 12 and 13 cells appeared similar to recruitment in purified day 7 and 8 cells, the appearance of A2B5(+) cells in long-term monolayers differed markedly. A2B5(+) cells that appeared in purified cultures for the most part did not have a neuronal morphology. They exhibited a flattened glial morphology (Fig.3-4) after about a week in culture and rested atop the A2B5(-) flat cells and were then round after several more days or when the cultures had become confluent. The appearance of these A2B5(+) cells with nonneuronal morhpology in purified cultures was not prevented by adding back the cells that were removed by the microspheres (data not shown). A2B5(+) cells with nonneuronal morphology also appeared in cultures made from dissociated cells that were kept in methylcellulose for a day (Fig.3-4). This was in contrast to the appearance of A2B5(+) cells in unpurified monolayer cultures. These cells exhibited a purely neuronal morphology in vitro and not the nonneuronal morphologies observed in the purified cultures. Nonneuronal A2B5(+) cells were also absent from cultures made from cells recovered from the microspheres and this fraction appeared to contain the largest and most numerous aggregates of neurons (data not shown). Some A2B5(+) cells with neuronal morphology were observed in purified

Fig.3-4. *In vitro* development of A2B5(+) cells in monolayer cultures made from unpurified (a-c) and purified (d-f) cells. Shown are phase/ fluorescent pairs with the antibody staining shown on the right. A2B5(+) cells in unpurified cultures appeared solely as neurons after about a week (a) or 2 weeks (b) *in vitro*. In these cultures, the antibody 5A11 reacted only with the flat A2B5(-) glia (c). In purified cultures, the A2B5(+) cells appeared predominantly with a flat glial morphology after about a week in culture (d) and were rounded up after several more days or when the monolayers approached confluency (e). Some A2B5(+) neurons with processes also developed in these cultures. The A2B5(+) nonneuronal cells reacted with 5A11 (f, arrowheads) revealing a partial glial phenotype. The micrograph pair in (g) shows several A2B5(+) nonneuronal cells in a monolayer culture that was made from unpurified cells that were suspended in methyl cellulose medium for 24 hours prior to plating. Thus these cells can be generated in culture by 2 different isolation procedures. Bar, 50μm.


a
b
c
d
e
f
g

cultures. These may have been the small number of NF(+)/A2B5(-) neurons that were detected in the initially purified cells (see above). The A2B5(+) cells in purified (long-term) monolayer cultures with nonneuronal morphology reacted with the monoclonal antibody 5A11. This antibody is known to be specific for glia in retina (Linser and Perkins, 1987b) and similarly reacted only with the surfaces of A2B5(-) glia in the cultures of unpurified optic tectum cells (Fig.3-4). Purified monolayer cultures contained many 5A11(+) round cells. These were presumably the A2B5(+) round cells seen in duplicate cultures, but double-label analysis could not be performed because both A2B5 and 5A11 are an IgM. Thus the A2B5(+) cells in purified cultures not only developed a nonneuronal morphology in culture but expressed a surface antigen (5A11) that normally seems to be restricted to glia.

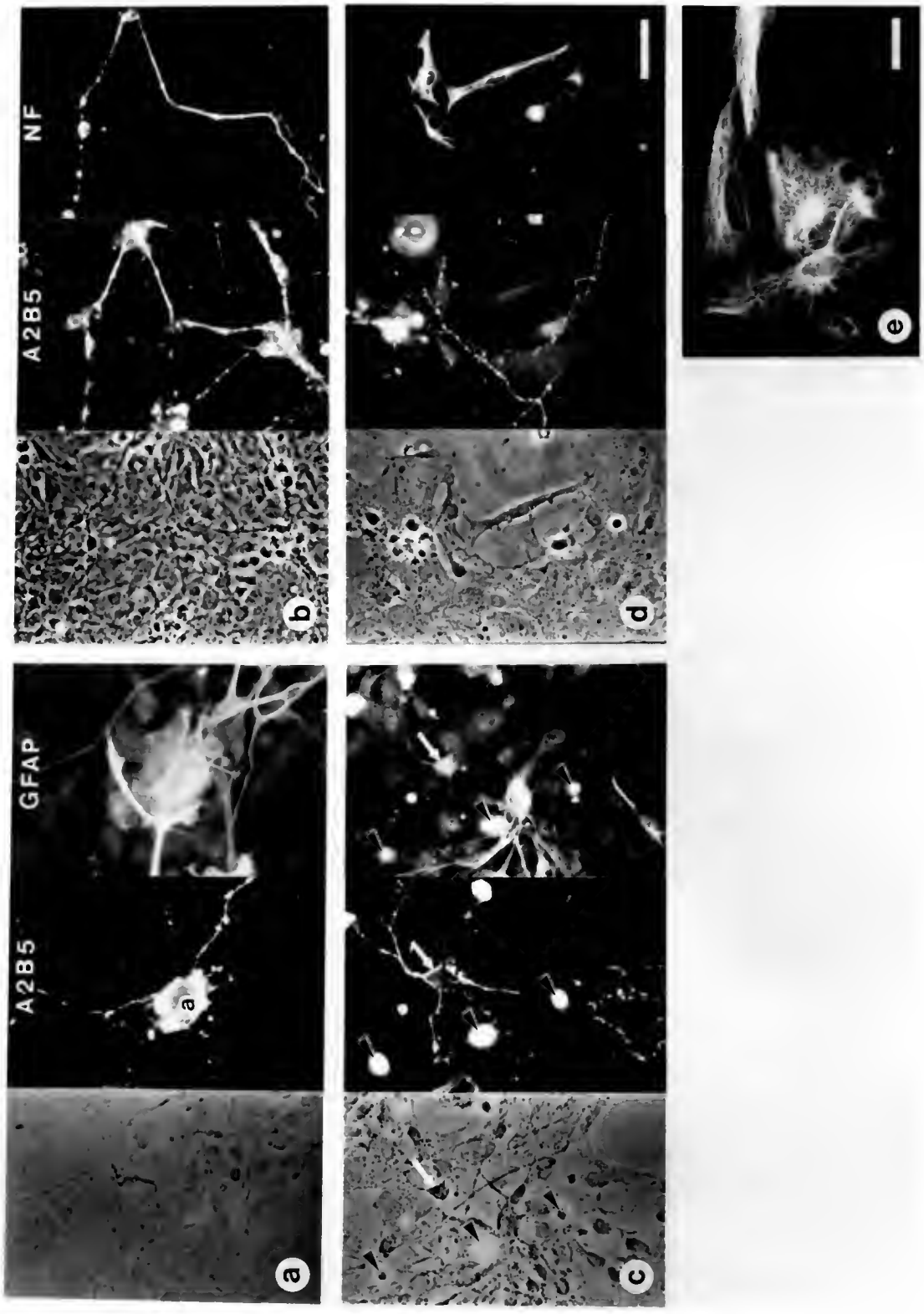
Intermediate Filament Expression In Vitro

Two types of intermediate filaments were investigated in cultures of unpurified and purified optic tectum cells. Glial filament expression was examined with both a commercial polyclonal antibody against bovine glial fibrillary acidic protein (GFAP) and monoclonal A5 (Debus et al., 1983). A5 did not react with anything in our cultures and so the following results are from using the commercial polyclonal antibody. In unpurified monolayer cultures GFAP reactivity was confined to the A2B5(-) flat

cells (Fig.3-5). Immunoreactivity was present in most of these cells. No reactivity was observed in the A2B5(+) neuronal cells. In purified cultures GFAP reactivity was also present in most of the A2B5(-) flat cells. Reactivity was also present as filaments, however, in many of the A2B5(+) flat and round cells that appeared in these cultures (Fig.3-5). So, not only do the A2B5(+) cells with nonneuronal morphology express the surface antigen recognized by 5A11 but some also appear to express glial filaments as well.

Neurofilament expression in cultures was examined with both a polyclonal antibody (NF-M) specific for the phosphorylated form of the middle weight neurofilament triplet protein and a monoclonal antibody (NF1) specific for the phosphorylated heavy weight triplet protein. In paraffin sections of tissue from 12 day tectum, NF-M reactivity was confined to neuronal processes and predominantly the 2 axonal layers, the stratum opticum and stratum album centrale (data not shown). In monolayer cultures made from unpurified cells, neurofilaments were observed in the processes and many cell bodies of A2B5(+) neurons. Not all NF-M(+) neurons were A2B5(+), however. Small numbers of neurofilament containing neurons were A2B5(-). An almost identical pattern of reactivity was exhibited by NF-M and NF1 in neuronal processes, although sometimes filaments that were NF-M(+) were not NF1(+). In

Fig.3-5. Expression of neurofilament reactivity (b,d,e) and glial filament reactivity (a,c) in 1-2 week old monolayer cultures of unpurified (a,b) and purified (c-e) cells. GFAP reactivity in unpurified cultures (a) was confined to the majority of flat A2B5(-) glia that underlie the networks of single and aggregated (labelled "a") neurons. In purified cultures (c) GFAP reactivity was also found in the A2B5(+) nonneuronal cells (arrowheads). An A2B5(+) cell with extended processes (white arrow) also appears to react with GFAP antibodies. Neurofilament reactivity in unpurified cultures (b) were present in neurons (mainly A2B5(+)) and not in the A2B5(-) glia. In purified cultures (d,e) NF reactivity also was found in some flat cells that were A2B5(-) or low level A2B5(+). Distinct filaments could be seen in these cells as shown in a higher magnification of several cells from a different field in (e). Bar (a-d), 50μm. Bar (e), 25μm.


A2B5
GFAP
A2B5
NF
a
b
c
d
e

purified cultures, NF-M(+) filaments were found in many of the A2B5(+) cells that had neuronal morphology (neurons). Surprisingly, many NF-M(+) flat and round cells were also observed dispersed throughout the culture (Fig.3-5). The NF-M reactivity was in the form of arrays of filaments. These cells were generally either low level A2B5(+) or A2B5(-). Immunoreactivity was faint or negative with NF1 for filaments in these cells. The other monoclonal antibodies specific for neurofilaments (DA3 and NN18) did not react with the monolayers, and so no double-labelling for GFAP and NF in these cells could be performed, because NF-M and the commercial anti-GFAP antiserum were both from rabbit. Anti-MSH which also reacts with the middle weight neurofilament triplet protein (Shaw et al., 1985) reacted with some neurons in monolayers similarly to NF-M, and also reacted with the A2B5(+) non-neuronal cells in purified cultures (not shown). The flat cells that contained neurofilament reactivity were not found in unpurified cultures.

Galactocerebroside Expression In Vitro

The development of oligodendrocytes *in vitro* was explored using a monoclonal antibody specific for the membrane marker galactocerebroside (GC; Ranscht et al., 1982). The GC(+) cells that initially were detected in dissociated and purified cells apparently developed into oligodendrocytes in culture. Numerous GC(+)

oligodendrocytes with multiple processes were found in both unpurified and purified long-term monolayers (Fig.3-6). As would correlate with the initial enrichment of GC(+) cells in purified cells, GC(+) oligodendrocytes were more numerous in purified monolayers. The oligodendrocytes were found primarily near clusters of neurons in both cultures, although there were many exceptions to this. All GC(+) cells that were observed were A2B5(-).

DNA Synthesis In Vitro

New DNA synthesis in the various cell types was investigated by ^{3}H-thymidine autoradiography combined with immunohistochemistry. This was done in order to determine if any patterns of new DNA synthesis could be discerned and correlated with particular cell phenotypes that appeared in culture. When freshly prepared cells were grown in medium containing ^{3}H-thymidine for 24 hours, a small number of unpurified cells contained labelled nuclei (Table 3-1). In 2 out of 3 experiments, purified cells also contained approximately equal small numbers of cells with labelled nuclei. In one experiment, however, the percent labelled nuclei of purified cells was several-fold that of the unpurified cells. In all instances, the cells that contained labelled nuclei were A2B5(-). Thus, the purified cells that became A2B5(+) by 24 hours did not synthesize new DNA in culture.

Fig.3-6. In vitro development of oligodendrocytes as defined by immunostaining with antibodies against GC. Shown are micrograph sets of cultures double-labelled for GC and A2B5. GC(+) oligodendrocytes developed in cultures from both unpurified (a) and purified (b) cells and were always A2B5(-). Oligodendrocytes frequently extended processes that were in close contact with A2B5(+) neurons (a). A large aggregate of neurons is demarcated by the "a". In purified cultures GC(+) oligodendrocytes were more numerous than in unpurified cultures. Shown in (b) is an oligodendrocyte in the same field as several A2B5(+) cells. Oligodendrocytes tended not to be in close proximity to A2B5(+) nonneuronal cells. Bar, 50μm.

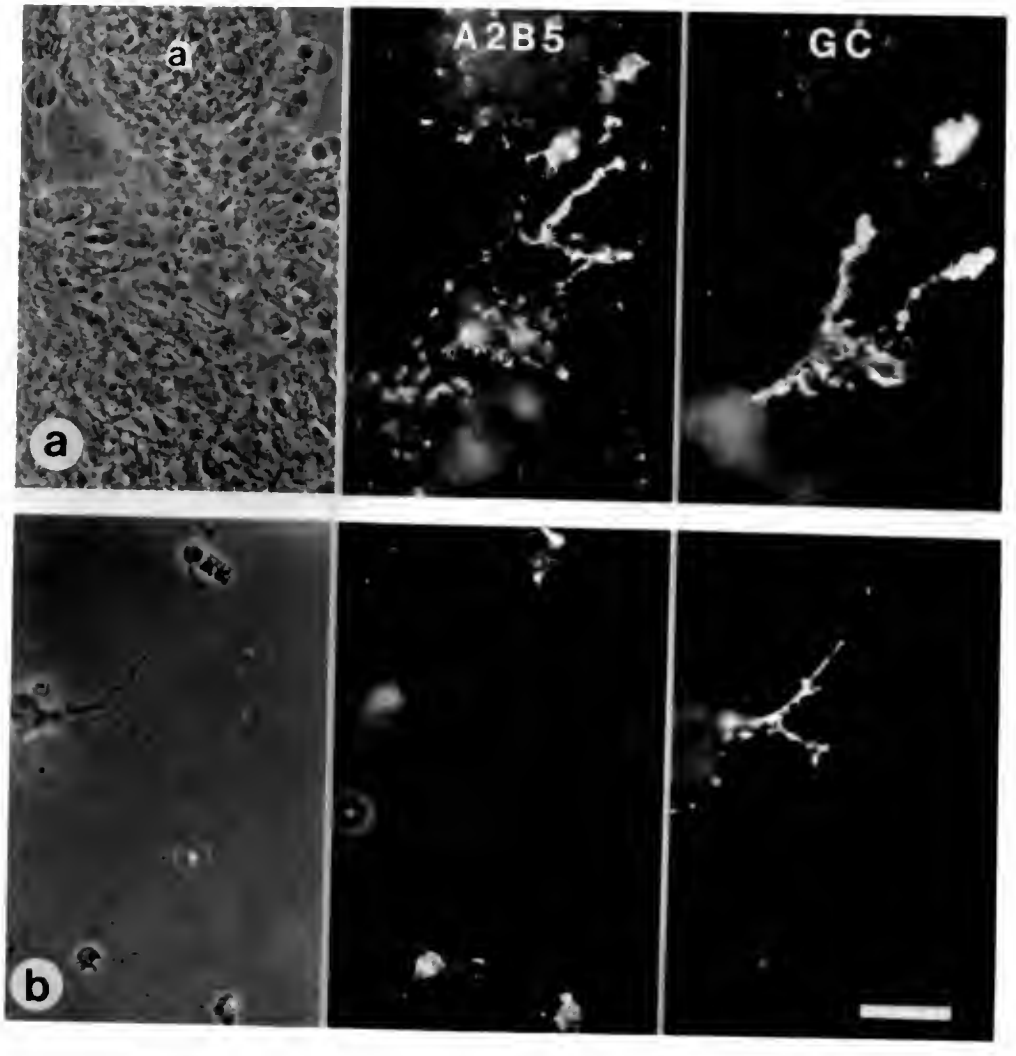

a
A2B5
GC
a
b

Table 3-1
^{3}H-thymidine incorporation:
Day 12-13 cells[a]

Cells		Score	% Labelled Nuclei	
Unpurified	1	11/315	3.5%	
	2	21/790	2.7%	mean=3.0±0.4
	3	9/326	2.8%	
Purified	1	33/722	4.6%	
	2	94/814	11.5%	mean=6.7±3.4
	3	15/383	3.9%	

[a] ^{3}H-thymidine incorporation into cells during 24 hours in culture. The results of 3 experiments are shown. In 2 of the 3 experiments (#1 and 3) both unpurified and purified cells contained a small percentage (<5%) of labelled nuclei. In 1 experiment (#2) the purified cells contained a much higher percentage of labelled nuclei (4x) than did unpurified cells. In all cases, labelled nuclei were in A2B5(-) cells. Newly recruited A2B5(+) cells in purified cultures therefore did not require new DNA synthesis for this change in phenotype.

Monolayer cultures were exposed to medium that contained ^{3}H-thymidine for the life of the culture (≈1½ weeks). In both unpurified and purified monolayers, the majority of the A2B5(-) flat cells contained labelled nuclei (Fig.3-7). In unpurified monolayers, A2B5(+) neurons were not labelled. Similarly, GC(+) oligodendrocytes did not contain labelled nuclei. In purified monolayers, neither GC(+) oligodendrocytes nor the A2B5(+) flat cells contained labelled nuclei. Thus, the only cell phenotype that synthesized new DNA in monolayer cultures was the A2B5(-) flat cell. These results are presented in tabular form in Table 3-2.

Discussion

A summary diagram of the results that includes cell phenotypes, approximate percentages of cells, and new DNA synthesis is presented in Fig. 3-8. The work presented here is an investigation of the *in vitro* development of dissociated and immunomagnetically purified 12 and 13 day embryonic chick optic tectum cells. The cell purification method that was used resulted in extremely purified populations of cells that were negative for the target cell monoclonal antibody A2B5. It was necessary to utilize a double protease treatment prior to the disruption of tissue into single cells with the day 12 and 13 tissue that was used. This was not necessary when less developed day 7 or 8 tissue was used (Chapter 2) and is believed to reflect the

Fig.3-7. ^{3}H-thymidine autoradiography combined with immunohistochemistry of monolayer cultures after 8 days *in vitro*. Cultures were continuously incubated in culture medium containing label, and dipped slides were exposed for 5 days before developing. Micrograph sets are comprised of (from left to right) phase contrast, brightfield, A2B5 immunostaining, and (a only) GC immunostaining. All black is autoradiographic label in the unstained brightfield micrographs, and nuclei appeared as large densely labelled areas. In both unpurified and purified cultures labelled nuclei were present only in A2B5(-) flat glia. A2B5(+) neurons (a), A2B5(+) nonneuronal cells (b), and GC(+) oligodendrocytes (a) did not contain labelled nuclei. Bar, 50μm.

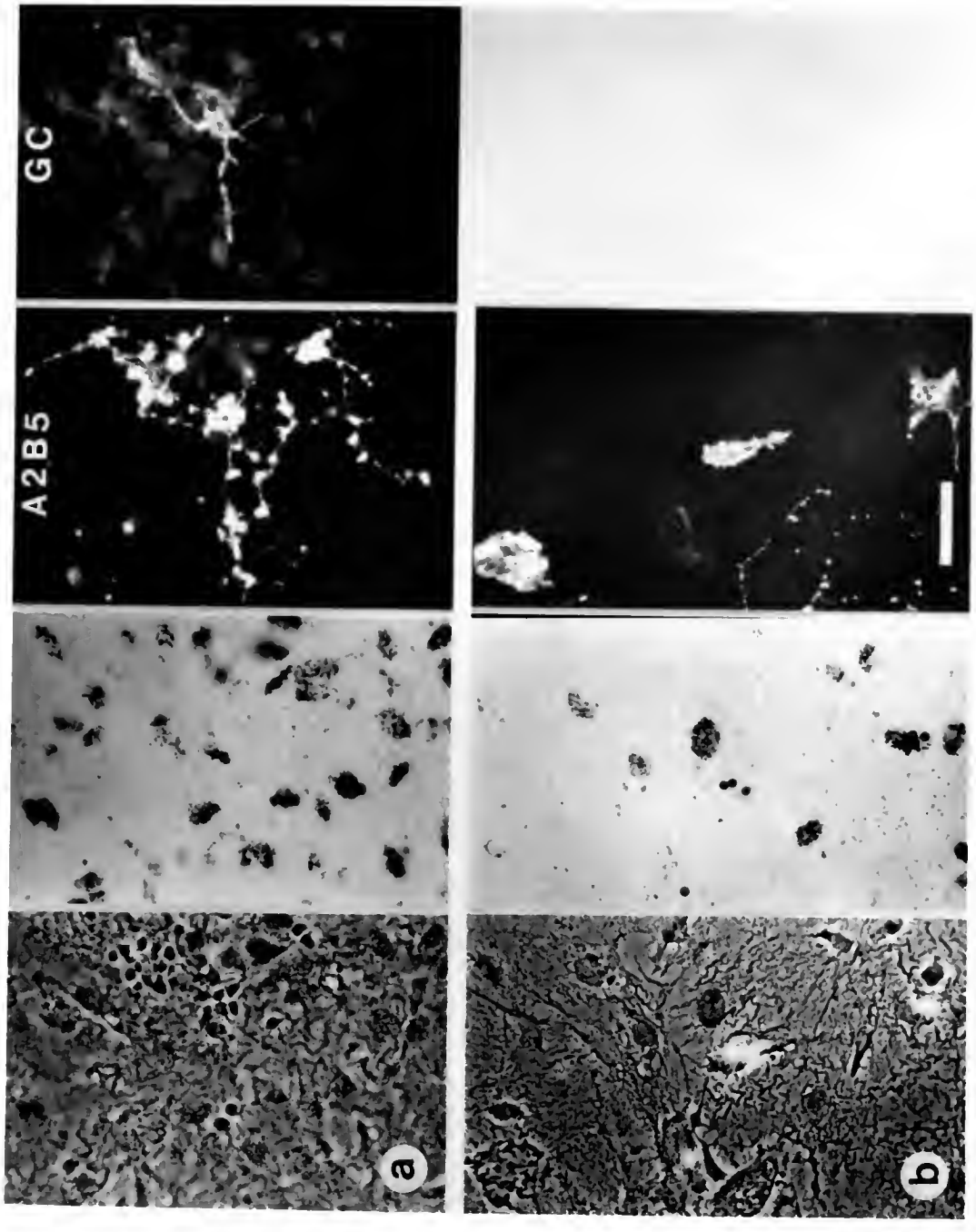
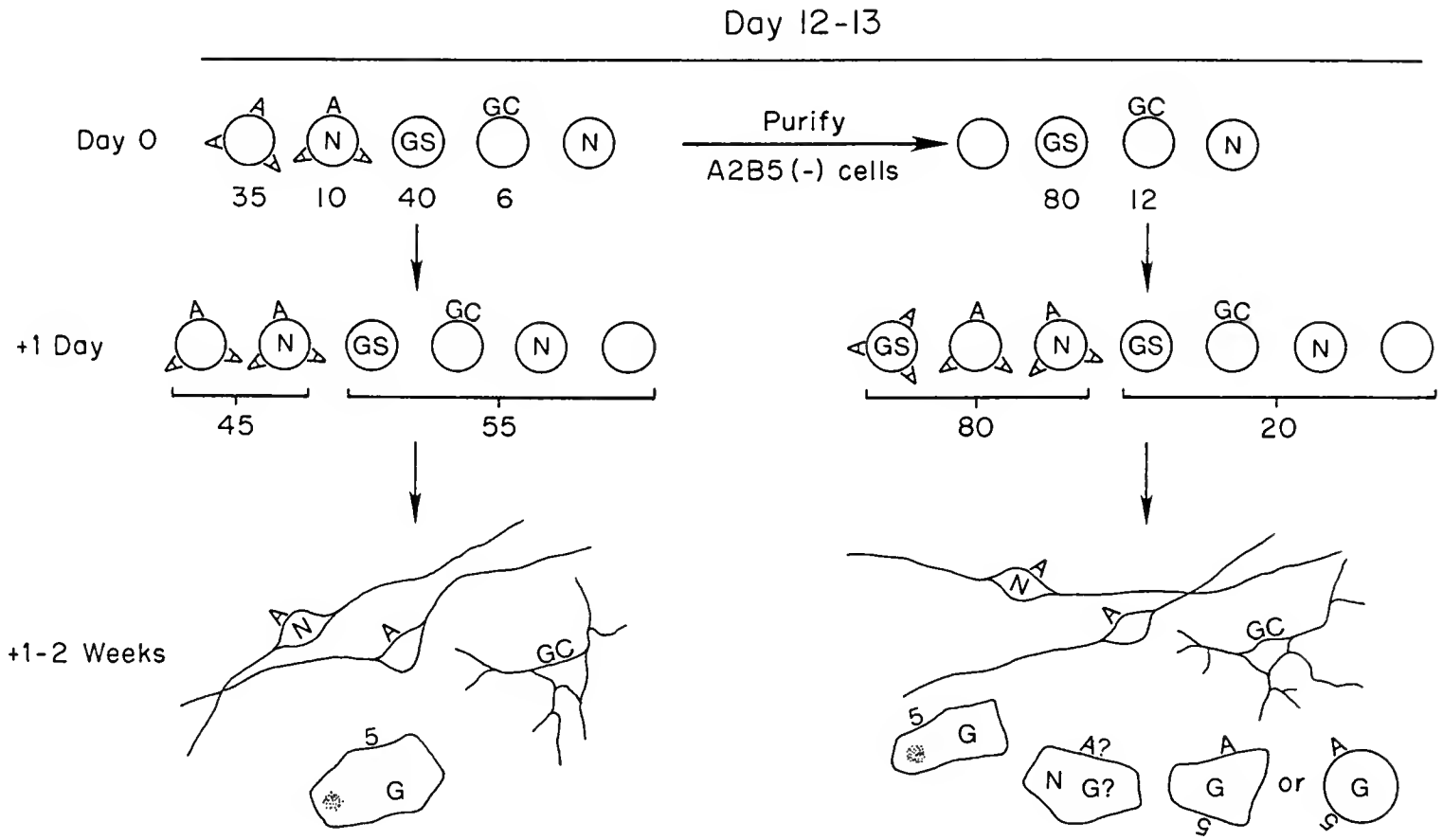

GC
A2B5
a
b

Table 3-2
^{3}H-thymidine incorporation:
Monolayers[a]

Cell Type	Marker	Labelled Nuclei
Flat	GFA/5A11	+
Neuron	A2B5/NF	-
Oligodendrocyte	GC	-
Abnormal	A2B5/GFA/ 5A11/NF	-

[a] ^{3}H-thymidine incorporation into monolayer cultures exposed to continuous labelling. Of the cell phenotypes described below, the only one that contained labelled nuclei was the A2B5(-) flat cell. This demonstrates that new DNA synthesis is not required for either recruitment of A2B5(+) cells from A2B5(-) cells or their differentiation. Similarly, cells that developed into neurons or oligodendrocytes did not need new DNA synthesis in culture.

Fig.3-8. Diagrammatic summary of the results which shows immuno-phenotype, morphology, and ^{3}H-thymidine incorporation of cells in unpurified (left) and purified (right) cultures. Time in culture is denoted on the left. The numbers below the cells indicate percentages of either unpurified (left) or purified (right) cells. "A" on a cell indicates cell surface A2B5 binding, "5" indicates binding of antibody 5A11, "GC" indicates galactocerebroside reactivity, "N" indicates neurofilament reactivity, "G" indicates glial fibrillary acidic protein reactivity, and "GS" indicates glutamine synthetase reactivity. Labelled nuclei are shown as intracellular stiplings.


Day 12-13
Day 0
A
A
N
GS
GC
N
35
10
40
6
Purify
A2B5 (-) cells
GS
GC
N
80
12
+1 Day
A
A
N
GS
GC
N
45
55
GS
A
A
N
GS
GC
N
80
20
+1-2 Weeks
A
N
A
GC
5
G
N
A
A
GC
5
G
A?
N
G?
A
G
5
or
A
G
5

more elaborate processes and apparently tighter adhesion between cells in the older tissue. This procedure resulted in smooth surfaced round cells which were required for optimum separations. An incomplete dissociation resulted in considerable trapping of nontarget cells that otherwise would have eluted.

Markers for Dissociated Cells

Day 12 marks the beginning of the growth and maturation phase in chick optic tectum (LaVail and Cowan, 1971a). With day 12 and 13 optic tectum cells, two widely used glial markers reacted with discrete populations of cells. Glutamine synthetase antibodies (Linser and Moscona, 1979) reacted with a large percentage of dissociated cells (≈40%) and the majority of purified cells (≈80%) identifying them as astroglia. The fact that these cells were A2B5(-) further supports the belief that A2B5 is neuron-specific in chick optic tectum at this developmental age. The oligodendrocyte marker galactocerebroside (Ranscht et al., 1982) was also found to react with a discrete population of dissociated cells (≈6%) and similarly these cells were all A2B5(-) and enriched for in the purified population (≈12%). Thus, the purified cells could be positively identified as either astrocytes or oligodendrocytes to a level of >90%.

Although more cells could be identified with glial markers, antibodies against neurofilaments were less useful

as a marker for neurons than at day 7 or 8 (see Chapter 2). Day 12 and 13 dissociated cells did not contain the easily discernable rings of neurofilaments that were characteristic of the day 7 and 8 cells. Most NF-M reactivity was in the form of small blebs on one side of the cell. These were presumably remnants of axons that were sheared off during tissue dissociation. This difference in immunoreactivity encountered in the older dissociated cells possibly reflected more stable attachments between neurons and thus an inability to retract their axons. Day 7 and 8 cells are in a phase of migration (LaVail and Cowan, 1971a) and therefore might not have had the stable attachments of the older tissue.

A2B5 Antigen and its Modulation

Purified cells were initially devoid of A2B5(+) cells. This was demonstrated by both the immunofluorescent as well as the immunoradiometric assays immeadiately following the separations. The appearance of A2B5(+) cells in the purified cell population *in vitro* was similar to that reported with day 7 and 8 cells and was similarly confirmed by the immunoradiometric assay. This suggests that the same mechanism may be operating for the recruitment of additional A2B5(+) cells with day 12 and 13 cells as was operating with day 7 and 8 cells. A major difference, though, is the extent to which this occurs. Here, the percentage of A2B5(+) cells that appears after 24 hrs. in

culture (80%) far surpasses the levels in unpurified cultures (45%). This suggests that the population of reactive cells is not taken from a resting blast population, as it is unlikely that more resting blast cells would be present after the phases of proliferation and migration within the tectum have ceased (LaVail and Cowan, 1971a,b) than during the proliferative phase. Alternatively, blast cells may have been released more easily from the tissue during dissociation than were differentiating cells. More importantly, since >90% of the purified cells were identified as definitive glia already expressing differentiation markers the reactive population must have come largely from definitive glia. This also demonstrates that, in the chick system, A2B5 antigen on the surfaces of glia was induced experimentally. And since the GC(+) cells in the purified population did not become A2B5(+) it appears that the reactive cells are wholly of the astrocyte lineage. Lastly, it is worth noting that none of the recruited A2B5(+) cells incorporated ^{3}H-thymidine. This clearly demonstrated that new DNA synthesis, and hence mitosis, was not required for A2B5 antigen modulation. Possible explanations of the mechanisms that may be operating to effect this change in phenotype are given in the Discussion section in Chapter II.

Development of Purified Cells In Vitro

The appearance of the A2B5(+) cells in purified long-term monolayer cultures made from day 12 and 13 cells differed markedly from that of A2B5(+) cells from day 7 and 8 (Chapter 2). Instead of appearing to be neurons, the majority of A2B5(+) cells exhibited a flattened glial and then round morphology. They also expressed the glial antigens GFAP and 5A11 in monolayer culture. These combinations of antigen expression (A2B5 with 5A11 or GFAP) were not found in unpurified cultures and are believed to be abnormally induced by the purifications. Likewise, in mouse cerebellar cultures other workers have experimentally induced the expression of A2B5 antigen on the surfaces of GFAP containing astrocytes as a result of neuronal depletion by complement mediated lysis using an independent neuronal marker (Nagata et al., 1986). Thus, the presence of A2B5 antigen on vertebrate astrocytes which have been in culture for even short periods of time (hours) should not be considered characteristic of a normal phenotype without reservation due to the apparent ease with which the antigen can be modulated.

Another phenotype that was presumably artifactually induced as a result of the purifications was flat cells containing dense networks anti-neurofilament-M reactive filaments. Some of these cells appeared to exhibit low level surface A2B5 reactivity, but the majority appeared to

be A2B5(-). The polyclonal antibody that was used (anti-NF-M) was well characterized (Bennett et al., 1984; Bennett and Dilulo, 1985) and was found not to react with other types of intermediate filaments on Western blots. In addition, most of the other flat cells that contained glial filaments as evidenced by reactivity with antibodies against GFAP did not exhibit reactivity with NF-M. These results indicate that the NF-M(+) reactivity in these cells represents the presence of neurofilament reactivity and not cross-reactivity with, say, GFAP filaments. The fact that the NF-M(+) flat cells did not show reactivity with the monoclonal antibody NF1 which is specific for the heavy weight triplet protein was curious. This may be explained by the results of others who have demonstrated that the expression of the 3 triplet proteins in chicks is not necessarily coordinate (Bennett et al., 1984; Dahl and Bignami, 1986) and that the last triplet protein to be expressed in phosphorylated form is often the heavy weight one (Dahl and Bignami, 1986; Dahl et al., 1986). In any case, the presence of neurofilament reactivity in cells that did not acquire a neuronal morphology is unique.

The lack of new DNA synthesis in all cells that were A2B5(+) was absolute. In purified cultures this negative correlation indicates several things. The first is that the recruited A2B5(+) cells at 1 day in culture did not require new DNA synthesis for the acquisition of cell surface A2B5

antigen. Also, the A2B5(+) cells did not require new DNA for their differentiation, whether it was into apparently normal neurons or into cells that also expressed GFAP and/or 5A11. In this respect these cells were like neurons (post-mitotic) and not the majority of A2B5(-) flat cells that were apparently glia and proliferated in culture. The lack of labelled nuclei in oligodendrocytes also indicates that the only cell population that may have proliferated in culture was the astrocytes.

The possibility of the existence of "type 2" astrocytes (Raff et al., 1983a) in the cultures must be discussed. These cells express both GFAP and A2B5 antigen and are thought to be a normal cell phenotype in vivo. To my knoweldge, these cells have never been documented in the chick system. Even in the rat system where they have been reported they are peculiar to the white matter fiber tracts, optic nerve and corpus callosum, and were not found in the brain (Raff et al., 1983b). Furthermore, when A2B5(+) cells were lysed via complement in that system, no new A2B5(+) cells appeared in culture. GC(+) cells were found to develop on time in cultures made from dissociated rat brain cells 13-14 days before they normally appeared in vivo (Abney et al., 1981). But, GC(+) oliodendrocytes did not develop in cultures of purified A2B5(-) cells from day 13-15 cells (Abney et al., 1983). Thus, the rat system appears to be different from chick in several respects.

However, in view of our results and others (Nagata et al., 1986) which indicate that A2B5 antigen may be modulated by loss of neuronal contact it seems possible that its expression on the surface of type 2 astrocytes may also be in response to neuronal deprivation. This would occur when the optic nerve was dissected as only axons are present there. By the time that the nerve is dissociated and the cells are plated several hours would have passed which in my system is ample time for many A2B5(-) cells to express the antigen on their surfaces (data not shown). This possibility would not be easy to rule out since I and others (Schnitzer and Schachner, 1982; Dr. M.F.Marusich, personal communication) have found that all neural cells, including glia, contain intracellular epitopes for A2B5. This last point precludes the usefulness of A2B5 as a neuronal marker in tissue that was first fixed and permeablized before incubation in A2B5, such as in tissue sections.

Explanation of Abnormal Phenotypes

The phenotypes that appeared in purified cultures such as A2B5(+)/5A11(+) round cells, NF-M(+) flat cells, and A2B5(+)/GFAP(+) flat cells were clearly abnormal. These markers that were co-localized in purified cultures were always segregated into different cells in unpurified cultures. The possibility that these abnormal phenotypes were generated as a result of the procedure itself seems

unlikely. Cells trypsinized from the microspheres did not produce them when placed in culture (not shown). The peculiar cells were also absent from eluted cultures that were not made from highly purified cells due to ineffective antibody coating of the microspheres (not shown). Separations of day 12-13 cells were also performed after dissociation of tissue with papain (not shown). This also resulted in A2B5(+) flat cells in the purified but not the unpurified cultures. Similarly, day 7-8 cells were separated after tissue dissociation with either papain or dispase. This resulted in A2B5(+) cells in purified cultures with neuronal morphologies, as was the result with dissociation after trypsin alone (see Chapter II). Thus, the presence of A2B5(+) flat cells in purified cultures is not believed to be a result of using a different dissociation protocol than with day 7-8 cells. However, the different age embryonic cells may react differently to the different proteases and, so, this possibility cannot be ruled out completely.

The fact that adding back microsphere fraction cells to the purified cells did not prevent the appearance of the A2B5(+) cells with nonneuronal morphology may have been due to their inability to communicate after separation. This may have been due to an alteration of the cell surfaces caused by the binding and/or removal of the cells from the microspheres. Alternatively, it may have been a reflection

of a mechanism that was simply rapid and irreversible. It is not known which of these or other possibilities had occured.

Just exactly what these abnormal phenotypes that appear in purified cultures represent is unclear. Several possibilities exist. One possibility is that they represent changes in astrocyte phenotypic characteristics which have been shown to be modulated by neuronal contact. Many instances of this type of phenomena have been documented in the past several years such as modulation of marker profiles (Fischer et al., 1986; Fisher, 1984; Holton and Weston, 1982; Linser and Perkins, 1987a; Nagata et al., 1986) and proliferation (Fischer et al., 1986; Hatten, 1987; Sobue and Pleasure, 1984).

Another possibility, however, is that these cells were reacting to neuronal depletion by attempting to switch cell types to become neurons. The range of mixed phenotypes would represent a continuum of transition states from glia to neurons. The phenotypes that exhibited more neuronal character represented cells that were able to more fully, but not totally, complete the attempted conversion from glia to neurons. The cells that exhibited more glial character represented those cells that had a more restricted potential and less ability to convert to neurons. This hypothesis is supported by the results of the previous chapter in which A2B5(+) cells in purified

monolayer cultures from younger day 7 and 8 tissue appeared to be normal neurons. While none of these possibilities can be settled upon, the drastic change in phenotype *in vitro* of purified A2B5(-) cells is further evidence of the plasticity of embryonic glial cells and their capacity to change in response to changes in their relationships with neurons.

CHAPTER IV
CONCLUSIONS

A diagrammatic summary of the results of separations of day 7-8 tectum cells is shown in Fig. 2-12. From the results presented here it is concluded that A2B5 is an accurate marker for neurons in long-term monolayer cultures made from unpurified embryonic chick OT cells. The immunomagnetic separation procedure used is an extremely effective technique for the separation of embryonic neural cell types based on cell surface antibody binding. Immunomagnetic depletion of A2B5(+) cells, and possibly neurons, from dissociated embryonic tectum cells resulted in recruitment of new A2B5(+) cells from cells that otherwise would not have been recruited.

Recruited cells from day 7 or 8 tissue compensated for this depletion by presumably developing a purely neuronal phenotype <u>in vitro</u>. The deleterious effect of polyornithine substrata on recruited cells suggests that the recruited cells were not initially neuronal and that the acquisition of the neuronal phenotype was not a single step process. New DNA synthesis was not required for A2B5 antigen modulation, nor for neuronal development. It is believed that it is not possible to purify day 7 or 8 glioblasts that remain as such unless the A2B5(-) cells that are

recruited to become neurons are a set population and a cell surface marker can be found to remove them as well. Future experiments should be aimed at this question.

One such experiment would be to perform a double tandem separation. Between separations enough time would be allowed so that recruitment of the A2B5(-) cells to A2B5(+) ones could occur (hours). The A2B5(+) cells would then be removed from this population and the purified A2B5(-) cells would be analyzed for further recruitment. If no second wave of recruitment occured, then it would seem that the reactive population of cells was a discrete one. Then this may very well allow the purification of immature glia devoid of neurons that presumably would not produce any GS. Neurons could then be added back to the glia to elicit GS production on cue. This would obviously lead to a whole range of further experiments aimed at the mechanism controlling contact-mediated GS production.

A diagrammatic summary of the results obtained from the separation of day 12-13 tectum cells is presented in Fig.3-8. Purified A2B5(-) cells from this developmental age are comprised almost entirely of identifiable glia with a small number of identifiable neurons. A2B5(+) cells appeared in purified cultures, but long-term monolayers contained A2B5(+) cells with a nonneuronal morphology that also express glial antigens GFAP and 5A11. Flat cells that contained abundant neurofilament reactivity were also

present in purified cultures. Galactocerebroside(+) oligodendrocytes were present in dissociated cells from this age, were all A2B5(-), and apparently continued to grow in culture. Only a small percentage of cells, all A2B5(-), incorporated ^{3}H-thymidine during the first 24 hours in culture of either unpurified or purified cells. Continuous labelling of monolayer cultures resulted in labelling of the majority of A2B5(-) flat cells, and of no A2B5(+) neurons, no A2B5(+) nonneuronal cells, and no oligodenrrocytes. Thus, new DNA synthesis was not required for the recruitment of A2B5(+) cells or presumably their differentiation in culture.

It is concluded that the phenotypic instability of the recruited cells from day 12-13 tissue reflected an unsuccessful attempt at developing a neuronal phenotype in vitro. This most likely was due to a restricted developmental potential of the reactive cells.

REFERENCES

Abney, E. R., Bartlett, P. P., and Raff, M. C. (1981). Astrocytes, ependymal cells, and oligodendrocytes develop on schedule in dissociated cell cultures of embryonic rat brain. Dev. Biol., 83, 301-310.

Abney, E. R., Williams, B. P., and Raff, M. C. (1983). Tracing the development of oligodendrocytes from precursor cells using monoclonal antibodies, fluorescence-activated cell sorting, and cell culture. Dev. Biol., 100, 166-171.

Adler, R., Manthorpe, M., and Varon, S. (1979). Separation of neuronal and nonneuronal cells in monolayer cultures from chick embryo optic lobe. Dev. Biol., 69, 424-435.

Bennett, G. S., and DiLullo, C. (1985). Transient expression of a neurofilament protein by replicating neuroepithelial cells of the embryonic chick brain. Dev. Biol. 107, 107-127.

Bennett, G. S., Tapscott, S. J., DiLullo, C., and Holtzer, H. (1984). Differential binding of antibodies against the neurofilament triplet proteins in different avian neurons. Brain Res. 304, 291-302.

Blackburn, C. C., Swank-Hill, P., and Schnarr, R. L. (1986). Gangliosides support neural retina cell adhesion. J. Biol. Chem., 261, 2873-2881.

Campbell, G. LeM., Schachner, M., and Sharrow, S. O. (1977). Isolation of glial cell-enriched and-depleted populations from mouse cerebellum by density gradient centrifugation and electronic cell sorting. Brain Res. 127, 69-86.

Cowan, W. M., Martin, A. H., and Wenger, E. (1968). Mitotic patterns in the optic tectum of the chick during normal development and after early removal of the optic vesicle. J. Exper. Zool., 169, 71-92.

Dahl, D., and Bignami, A. (1986). Neurofilament phosphorylation in development. A sign of axonal maturation? Exper. Cell Res. 162, 220-230.

Dahl, D., Crosby, C. J., Gardner, E. E., and Bignami, A. (1986). Delayed phosphorylation of the largest neurofilament protein in rat optic nerve development. J. Neurosci. Res. 15, 513-519.

Debus, E., Weber, K., and Osborn, M. (1983). Monoclonal antibodies specific for glial fibrillary acidic (GFA) protein and for each of the neurofilament triplet proteins. Differ. 25, 193-203.

Eisenbarth, G. S., Walsh, F. S., and Nirenberg, M. (1979). Monoclonal antibody to a plasma membrane antigen of neurons. Proc. Natl. Acad. Sci. USA, 76, 4913-4917.

Fischer, G., Keilhauer, G., and Schachner, M. (1986). Comparison of astrocytic morphology, proliferation, marker profile, and response to neurons in wild-type and weaver mutant mouse cerebella in culture. Develop. Brain Res., 28, 247-257.

Fisher, M. (1984). Neuron-glia interactions and glial enzyme expression in mouse cerebellum. Adv. Exper. Biol. Med., 181, 203-213.

Folkman, J., and Moscona, A. (1978). Role of cell shape in growth control. Nature (London), 273, 345-349.

Fujita, S. (1964). Analysis of neuron differentiation in the central nervous system by tritiated thymidine autoradiography. J. Compar. Neurol., 122, 311-327.

Gammon, C. M., and Ledeen, R. W. (1985). Evidence for the presence of a ganglioside transfer protein in brain. J. Neurochem., 44, 979-982.

Gee, A.P., Lee, C., Bruce, K., Janssen, W., Graham-Poole, J., Pawlina, T., Pick, T., Harvey, W., Koch, P., and Gross, S. (1987). Immunomagnetic purging and autologous transplantation in stage D neuroblastoma. Bone Marrow Transplant., 2 (Suppl. 2), 89-93.

Grunwald, G. B., Fredman, P., Magnani, J. L., Ginsburg, V., and Nirenberg, M. (1985). Monoclonal antibody 18B8 detects gangliosides associated with neuronal differentiation and synapse formation. Proc. Natl. Acad. Sci. USA, 82, 4008-4012.

Hamberger, A., and Svennerholm, L. (1971). Composition of gangliosides and phospholipids of neuronal and glial cell enriched fractions. J. Neurochem., 18, 1821-1829.

Hatten, M. E. (1987). Neuronal inhibition of astroglial proliferation is membrane mediated. J. Cell Biol., 104, 1353-1360.

Hefti, F., Hartikka, J., and Frick, W. (1985). Gangliosides alter morphology and growth of astrocytes and increase the activity of choline acetyltransferase in cultures of dissociated septal cells. J. Neurosci., 5, 2086-2094.

Holton, B., and Weston, J. A. (1982). Analysis of glial cell differentiation in peripheral nervous tissue: II. neurons promote S100 synthesis by purified glial precursor cell populations. Develop. Biol., 89, 72-81.

Honig, M. G., and Hume, R. I. (1986). Fluorescent carbocyanine dyes allow living neurons of identified origin to be studied in long-term cultures. J. Cell Biol., 103, 171-187.

Hunter, W. M. (1978). Radioimmunoassay. In "Handbook of Experimental Immunology. Volume 1: Immunochemistry" (D. M. Weir, Ed.), pp. 14.1-14.40. Blackwell Scientific Publications, Oxford, England.

Kasai, N., and Yu, R. (1983). The monoclonal antibody A2B5 is specific to ganglioside G_{Q1c}. Brain Res., 277, 155-158.

Kelly, J. P., and Cowan, W. M. (1972). Studies on the development of the chick optic tectum. III. Effects of early eye removal. Brain Res., 42, 263-288.

Kim, S. U. (1985). Antigen expression by glial cells grown in culture. J. Neuroimmunol., 8, 255-282.

Kim, S. U., Moretto, G., Lee, V., and Yu, R. K. (1986). Neuroimmunology of gangliosides in human neurons and glial cells in culture. J. Neurosci. Res., 15, 303-321.

Landa, C. A., Maccioni, H. J. F., and Caputto, R. (1979). The site of synthesis of gangliosides in the chick optic system. J. Neurochem., 33, 825-838.

LaVail, J. H., and Cowan, W. M. (1971a). The development of the chick optic tectum. I. Normal morphology and cyto-architectonic development. Brain Res., 28, 391-419.

LaVail, J. H., and Cowan, W. M. (1971b). The development of the chick optic tectum. II. Autoradiographic studies. Brain Res., 28, 421-441.

Ledeen, R. (1985). Gangliosides of the neuron. Trends in Neurosci., 8, 169-174.

Ledig, M., Tholey, G., and Mandel, P. (1985). Factors involved in expression of neuron-specific and non-neuronal enolase activity in developing chick brain and in primary cultures of chick neurons. Develop. Brain Res., 21, 107-113.

Le Lievre, C. S., Schweizer, G. G., Ziller, C. M., and Le Douarin, N. M. (1980). Restrictions of developmental capabilities in neural crest cell derivatives as tested by in vivo transplantation experiments. Dev. Biol., 77, 362-378.

Levitt, P., and Rakic, P. (1980). Immunoperoxidase localization of glial fibrillary acidic protein in radial glial cells and astrocytes of the developing rhesus monkey brain. J. Comp. Neurol., 193, 417-448.

Linser, P. J. (1985). Multiple marker analysis in the avian optic tectum reveals three classes of neuroglia and carbonic anhydrase-containing neurons. J. Neurosci., 5(9), 2388-2396).

Linser, P. J. (1987). Neuronal-glial interactions in retina development. Amer. Zool., 27, 161-169.

Linser, P. J., and Moscona, A. A. (1979). Induction of glutamine synthetase in embryonic neural retina: Localization in Muller fibers and dependence on cell interactions. Proc. Natl. Acad. Sci. USA, 76, 6476-6480.

Linser, P. J., and Moscona, A. A. (1983). Hormonal induction of glutamine synthetase in cultures of embryonic retina cells: Requirement for neuron-glia interactions. Dev. Biol., 96, 529-534.

Linser, P. J., and Perkins, M. (1987a). Gliogenesis in the embryonic avian optic tectum: Neuronal-glial interactions influence astroglial phenotypic characteristics. Dev. Brain Res., 31, 277-290.

Linser, P. J., and Perkins, M. (1987b). Regulatory aspects of the in vitro development of retinal Muller glial cells. Cell Differ., 20, 189-196.

Meier, D. H., Lagenaur, C., and Schachner, M. (1982). Immunoselection of oligodendrocytes by magnetic beads. I. Determination of antibody coupling parameters and cell binding conditions. J. Neurosci. Res., 7, 119-134.

Meier, D., and Schachner, M. (1982). Immunoselection of oligodendrocytes by magnetic beads. II. In vitro maintenance of immunoselected oligodendrocytes. J. Neurosci. Res., 7, 135-145.

Nagata, I., Keilhauer, G., and Schachner, M. (1986). Neuronal influence on antigenic marker profile, cell shape, and proliferation of cultured astrocytes obtained by microdissection of distinct layers from early postnatal mouse cerebellum. Dev. Brain Res., 24, 217-232.

Philip, T., Favrot, M., and Philip, I. (1987). Complement and magnetic purging: A comparison in the Burkitt lymphoma model. Bone Marrow Transplant., 2 (Suppl. 2), 78-80.

Politi, L., and Adler, R. (1987). Selective destruction of photoreceptor cells by anti-opsin antibodies. Invest. Ophthalmol. Vis. Sci. 28, 118-125.

Puelles, L., and Bendala, M. C. (1978). Differentiation of neuroblasts in the chick optic tectum up to eight days of incubation: A Golgi study. Neurosci., 3, 307-325.

Raff, M. C., Abney, E. R., Cohen, J., Lindsay, R., and Noble, M. (1983b). Two types of astrocytes in cultures of developing rat white matter: Differences in morphology, surface gangliosides, and growth characteristics. J. Neurosci., 3, 1289-1300.

Raff, M. C., Miller, R. H., and Noble, M. (1983a). A glial progenitor cell that develops in vitro into an astrocyte or an oligodendrocyte depending on the culture medium. Nature (London), 303, 390-396.

Ranscht, B., Clapshaw, P. A., Price, J., Noble, M., and Seifert, W. (1982). Development of oligodendrocytes and Schwann cells studied with a monoclonal antibody against galactocerebroside. Proc. Natl. Acad. Sci. USA, 79, 2709-2713.

Rosner, H. (1980). Ganglioside changes in the chicken optic lobes and cerebrum during embryonic development. Transient occurrence of "novel" multisialo -gangliosides. Wilhelm Roux's Archives, 188, 205-213.

Schnitzer, J., and Schachner, M. (1982). Cell type specificity of a neural cell surface antigen recognized by the monoclonal antibody A2B5. Cell Tiss. Res., 224, 625-636.

Shaw, G., Fischer, S., and Weber, K. (1985). Anti-MSH and neurofilament M-protein share a continuous epitope but not extended sequences. FEBS Letters, 181, 343-346.

Shaw, G., Osborn, M., and Weber, K. (1986). Reactivity of a panel of neurofilament antibodies on phosphorylated and dephosphorylated neurofilaments. Eur. J. Cell Biol. 42, 1-9.

Sheffield, J. B., Pressman, D., and Lynch, M. (1980). Cells isolated from the embryonic neural retina differ in behavior in vitro and membrane structure. Science, 209, 1043-1045.

Sobue, G., and Pleasure, D. (1984). Astroglial proliferation and phenotype are modulated by neuronal plasma membrane. Brain Res., 324, 175-179.

Treleaven, J. G., Ugelstad, J., Philip, T, Gibson, F. M., Rembaum, A., Caine, C. D., and Kemshead, J. T. (1984). Removal of neuroblastoma cells from bone marrow with monoclonal antibodies conjugated to magnetic microspheres. Lancet, 1, 70-73.

Turner, D. L., and Cepko, C. L. (1987). A common progenitor for neurons and glia persists in rat retina late in development. Nature, 328, 131-136.

Wu, D. K., Scully, S., and de Vellis, J. (1988). Induction of glutamine synthetase in rat astrocytes by co-cultivation with embryonic chick neurons. J. Neurochem., 50, 929-935.

Wysocki, L. J., and Sato, V. L. (1978). "Panning" for lymphocytes: A method for cell selection. Proc. Natl. Acad. Sci. USA, 75, 2844-2848.

BIOGRAPHICAL SKETCH

Deni S. Galileo was born in Pittsburgh, Pennsylvania, on November 23, 1961. He attended elementary school there until moving to Meadville, Pennsylvania in 1968. He attended the public schools there and graduated from Meadville Area Senior High School in 1979. Deni attended and finally graduated from New College, the honors college of the University of South Florida in Sarasota, in 1983 after being academically dismissed after his first term. Deni obtained a Bachelor of Arts degree in cell and developmental biology with his bachelor's thesis entitled "Investigations on primary mesenchyme cell migration in the sea urchin Lytechinus variegatus: a model system for studying selective cellular adhesion in vivo" under Dr. John B. Morrill. He entered graduate school the following year at the Department of Anatomy and Cell Biology, University of Florida to further his education in developmental and cell biology with Dr. Paul J. Linser at the Whitney Laboratory. He obtained the degree of Doctor of Philosophy in basic medical sciences from the College of Medicine in August, 1988. Deni has accepted a postdoctoral research position at Washington University School of Medicine in St. Louis, Missouri, to continue his studies of brain development in chicks using recombinant retroviral vectors with Dr. Joshua Sanes.

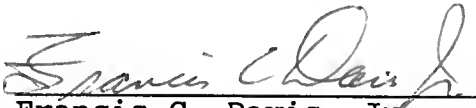
I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Paul J. Linser, Chairman
Associate Professor of Anatomy
and Cell Biology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

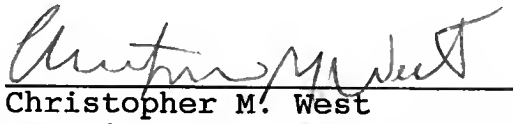
Carl M. Feldherr
Professor of Anatomy and Cell
Biology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



Francis C. Davis, Jr.
Associate Professor of
Microbiology and Cell Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



Christopher M. West
Associate Professor of Anatomy
and Cell Biology

This dissertation was submitted to the Graduate Faculty of the College of Medicine and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August, 1988

Dean, College of Medicine 7/6/88

Dean, Graduate School